PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Daniel JAVITT

Docket No: A8311

Appln. No.: 10/066,657

Group Art Unit: 1617

Confirmation No.: 5724

Examiner: Theodore J. Criares

Filed: February 06, 2002

For: D-SERINE TRANSPORT ANTAGONIST FOR TREATING PSYCHOSIS

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Daniel Javitt, hereby declare and state:

THAT I am a citizen of the United States of America;

THAT I have received an M.D. Degree in 1983 and a Ph.D. Degree in 1990 from the Albert Einstein College of Medicine.

THAT I have been employed by the Nathan Kline Institute since 1997, where I hold a position as Psyhiatrist III (research), with responsibility for Directorship of Program in Cognitive Neuroscience and Schizophrenia; and

THAT I am the inventor of the subject matter of the above-identified application and I am familiar with the proceedings in the prosecution of the present application.

I provide the following as evidence that the claims are enabled by the specification as originally filed and in support of the novelty and nonobviousness of the claimed invention in view of the prior art.

**D-serine transport inhibitors as new treatments for schizophrenia;
Preclinical Characterization – August 16, 2004**

Rationale

Schizophrenia is associated with disturbances in neurotransmission at *N*-methyl-D-aspartate (NMDA) type glutamate receptors that may be ameliorated by the administration of agents that augment NMDA receptor-medicated neurotransmission by stimulating the NMDA-associated glycine binding site. Endogenous ligands for this site include glycine and D-serine. Administration of these agents has been shown to effectively ameliorate persistent negative and cognitive symptoms of schizophrenia, supporting the underlying hypothesis. However, required doses of these agents are high, and D-serine has been associated with nephrotoxicity in rodents although not, to date, in primates. More refined approaches to stimulating the glycine/D-serine binding site of the NMDA receptor, therefore, may be desirable.

Glycine levels in brain are regulated by well described transport systems, including GLYT1 and SNAT2, which serve to maintain low glycine levels in the immediate vicinity of the NMDA receptor complex. Thus, suggesting that inhibition of these transport systems might lead to enhanced NMDA receptor-mediated neurotransmission. Preclinical data with prototypic glycine transport inhibitors, including GDA (1, 2), NFPS (3-5) and Org 25498 (6), as well as clinical findings with the non-selective glycine transport inhibitor sarcosine (7), support the role of glycine transport processes in NMDA regulation and schizophrenia treatment.

As compared with glycine transport processes, D-serine transport processes are relatively poorly described. As preliminary data for this project, we demonstrated the existence of a high-affinity, selective D-serine transport process in rat brain synaptosomes (8). Further, several

neuronally derived cell lines were identified that expressed endogenous D-serine transport, permitting their use in the screening of potential high affinity D-serine transport inhibitors that could then be used to evaluate functional importance of D-serine transporters in regulating processes of relevance to schizophrenia. This report describes initial preclinical results with 3 lead compounds determined from a screening library of 200+ D-serine derivative manufactured under contract by Albany Molecular Research, Albany, NY.

Compound Description

Initial structures were determined by analogy to high affinity inhibitors for GLYT1 and other amino acid transporters. The D-serine backbone was chosen as the starting point from synthesis. Hydrophobic derivative compounds were made by substation at the amino, carboxyl and alcohol positions of the D-serine molecule. Screening was done recursively, with substitutions producing a higher potency blockade which was retained in subsequent rounds of synthesis. The initial goal of this project was identification of compounds with $<10^{-5}$ M (100 μM) affinity for the D-serine transport process expressed either in cell culture or cortical synaptosomes. Three compounds were identified with requisite potencies, which were also amenable to scale up synthesis to quantities needed for *in vivo* investigation. Affinities of these compounds for inhibition of D-serine transport are shown in Table 1. Binding curves are shown

Table 1: IC_{50} values for inhibition of D-SERINE transport by prototype D-SERINE transport inhibitors: human neuroblastoma cells and cortical synaptosomes

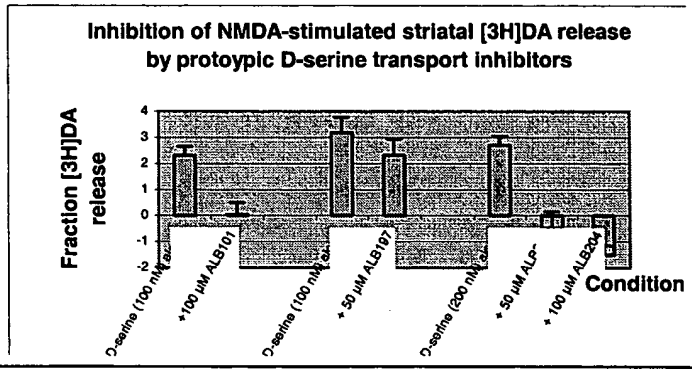
	cell culture (neuroblastoma)	Rat cortical synaptosomes
ALB101	16.3 μM (range: 3.46 - 77.1)	83.0 μM (range: 19.8 – 3485)
ALB197	7.3 μM (range: 2.2 – 23.9)	44.5 μM (range: 8.2 – 240.6)
ALB204	20.2 μM (range: 4.5 – 89.2)	264 μM (range: .017 – 4046)

in Appendix A. All compounds showed <50% inhibition of transport mediated via other neural transport systems at concentrations that substantially abolished high-affinity D-serine transport.

In vitro Dopamine Release

Initial screening was performed using an *in vitro* screening system that has previously been shown to be sensitive to effects of NMDA augmenting agents, including glycine and glycine transport inhibitors (9, 10). In this system, isolated mouse striatum is preincubated with [^{3}H]DA, which is incorporated into presynaptic dopaminergic stores. DA release is then initiated by brief exposure to NMDA. The effect of test agents on NMDA-stimulated DA release is measured. Schizophrenia, in general, is associated with hyperactivity of striatal DA release (11). A common action of glycine and glycine transport inhibitors, therefore, is inhibition of NMDA stimulated DA release (9, 10). The three lead compounds (ALB101, ALB197 and ALB204) were therefore screened for their ability to inhibit release. Assays were conducted in both the absence and presence of added D-serine. No statistical difference was found between the two conditions, although results appeared more consistent in the presence of low concentrations of D-serine (100-200 nM), potentially recreating the endogenous *in vivo* situation. All 3 lead compounds produced effects in the expected direction (Figure 1), with results being most robust for ALB101 (t=3.59, df=34, p<.0004) and ALB204 (50uM: t-4.84, df=28, p<.0001; 100 µM:

Figure 1: Effect of prototype D-SERINE transport inhibitors on NMDA stimulated DA release



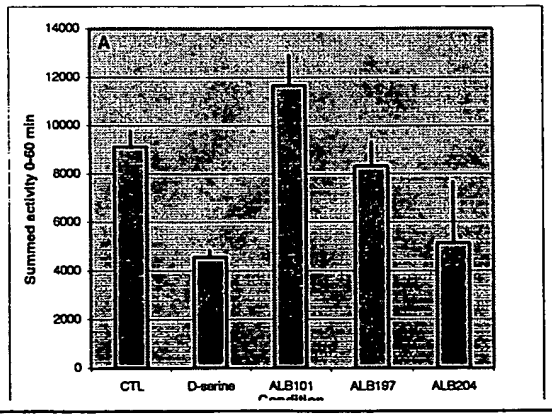
t=8.61, df=33, p<.0001). Mean response curves from the release assay experiment are shown in Appendix B.

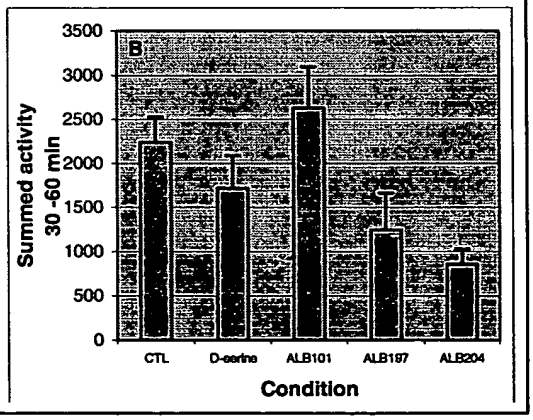
In vivo Behavior

Finally, in order to assess relative *in vivo* potency of prototypic glycine transport inhibitors, locomotor hyperactivity was evaluated in response to amphetamine or PCP challenge. In prior studies, we demonstrated that glycine and the prototypic glycine transport inhibitor GDA inhibited hyperactivity induced by PCP but not amphetamine (1), with similar reports being observed as well for the high affinity glycine transport inhibitors NFPS and Org 24461 (12). In these studies, effects of D-serine were compared to those of the prototype compounds ALB101, ALB197 and ALB204.

In our preliminary studies, a somewhat different profile of activity was observed for D-serine, with D-serine treatment leading to inhibition of both amphetamine- (Fig. 3A) and PCP (Fig. 3B)-induced hyperactivity at a dose of 25 μmol/g (=2625 mg/kg). Of these, only the effect

Table 3: Effect of D-SERINE **and prototype** D-SERINE **transport inhibitors on locomotor activity induced by amphetamine (*A*, left) and PCP (*B*, right)**



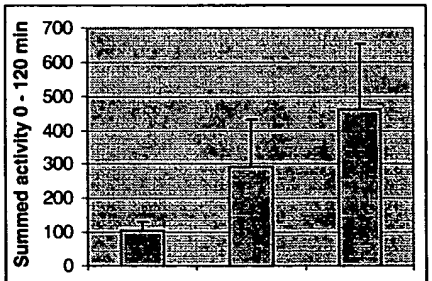


on amphetamine-induced hyperactivity proved statistically significant (t=5.54, df=107, p<.0001). The need for high doses of D-serine to reverse hyperactivity induced by NMDA antagonists is

consistent with previous reports in this area. In particular, Nilsson et al. (13) observed significant inhibition of MK-801-induced hyperactivity only at a dose of 4000 mg/kg. Significant effects in the expected direction were observed for prototypic D-serine transport inhibitors as a group. Results were again most significant for ALB204, which produced significant inhibition of both amphetamine (t=3.23, df=81, p=.002) and PCP (t=2.61, df=49, p=.012) induced hyperactivity at a dose of 100 mg/kg ip. This compound was thus approximately 25-fold more effective than D-serine in producing its behavioral effects. Activity curves are shown in Appendix C.

For initial studies with these compounds, Balb/c mice were used for PCP-induced hyperactivity studies because these mice are known to show greater locomotor hyperactivity to PCP than the more commonly used C57 mice (14). In order to verify, however, that effects of D-serine and ALB204 were not due to nonspecific locomotor depressant effects, studies with ALB204 were performed also in C57 mice. In these mice, we previously observed (unpublished observations) that D-serine produces a significant enhancement of locomotor activity following PCP administration, presumably reflecting reversal of PCP-induced hypoactivity similar to that observed in primates. In C57, mice, as we observed previously, D-serine produced increased locomotor activity (distance traveled) that was significant at trend level (t=1.79, df=21, p=.087). In contrast, ALB204 produced a robust increase in activity that was statistically significant (t=2.65, df=20, p=.016), and in the opposite direction from its effects in Balb/c mice. Thus, anti-PCP effects of ALB204

Figure 4: Effect of D-SERINE and ALB204 on PCP-induced activity – C57 mice



cannot be attributed to general locomotor depressant effects, and thus may be mediated by significant potentiation of NMDA receptor-mediated neurotransmission.

Conclusion

The present studies were performed with the goal of exploring feasibility of developing D-serine transport inhibitors as potential treatments for schizophrenia. The results of the parallel *in vitro* and *in vivo* investigations demonstrate first the "druggability" of the D-serine transport inhibitor site, with compounds from a screening library showing predictable inhibitory effects on D-serine transport; and second, that compounds targeting the D-serine transport site show predicted effects in models previously shown to be sensitive to effects of NMDA receptor agonists and antagonists. Of the compounds screened, ALB204 showed greatest *in vitro* and *in vivo* effectiveness and may be an appropriate compound for future, large scale drug development studies.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 12/17/2005

Dr. Daniel Javitt

References

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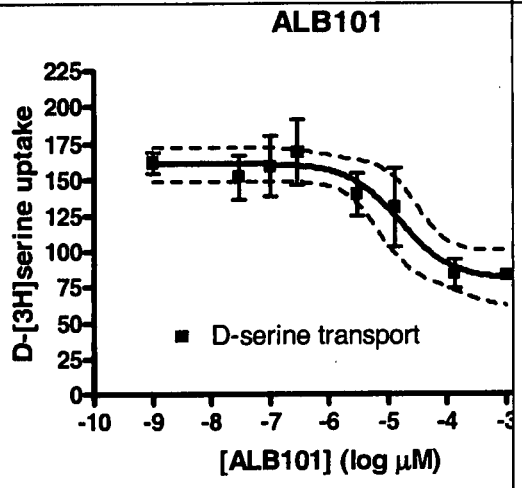
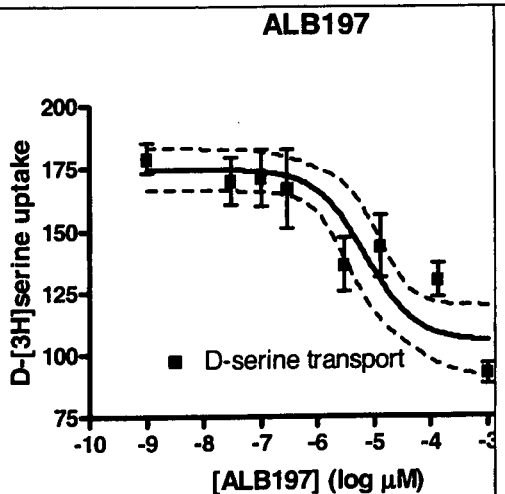
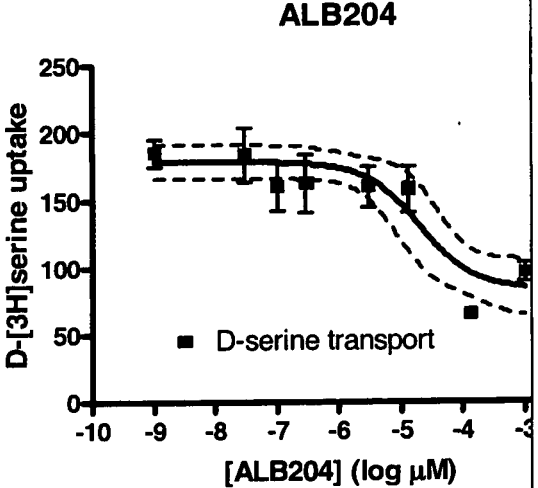
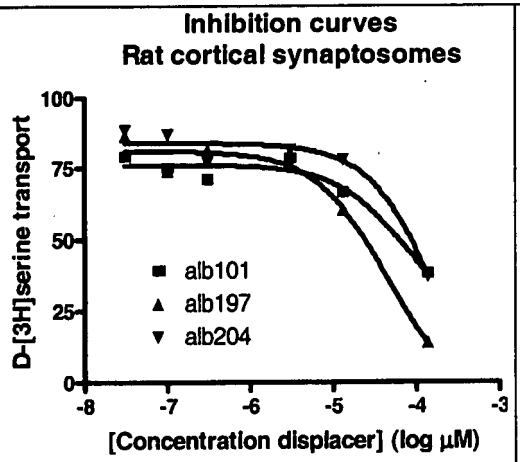
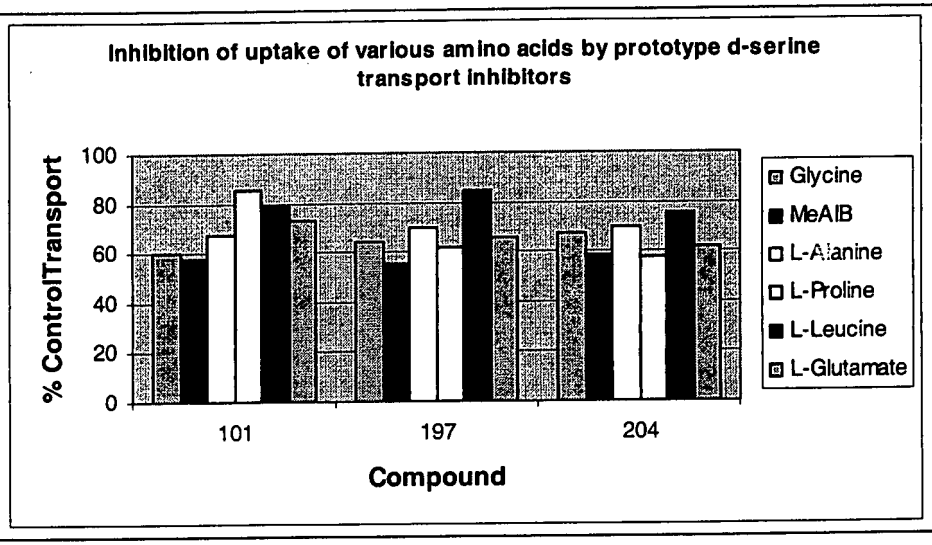
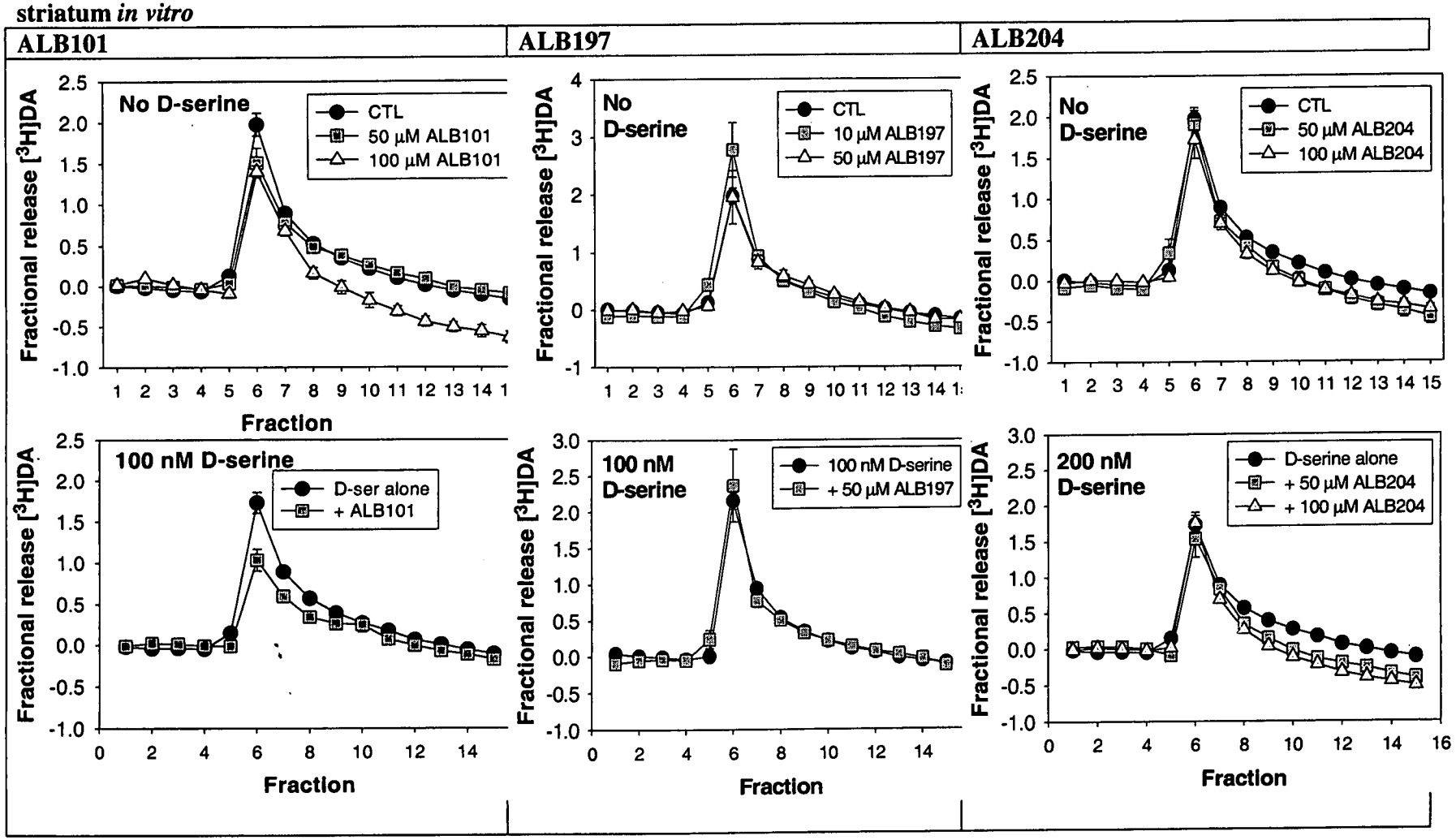
14. Toth E, Lajtha A. Antagonism of phencyclidine-induced hyperactivity by glycine in mice. Neurochem. Res. 1986;11:393-400.

Appendix A: Summary of transport studies with prototypic D-serine transport inhibitors

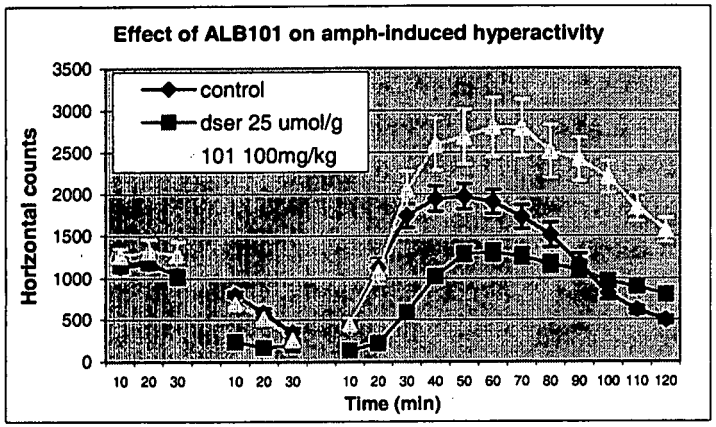
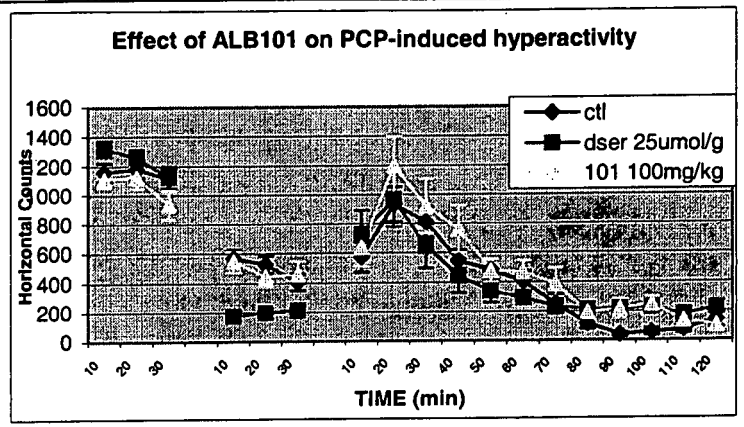
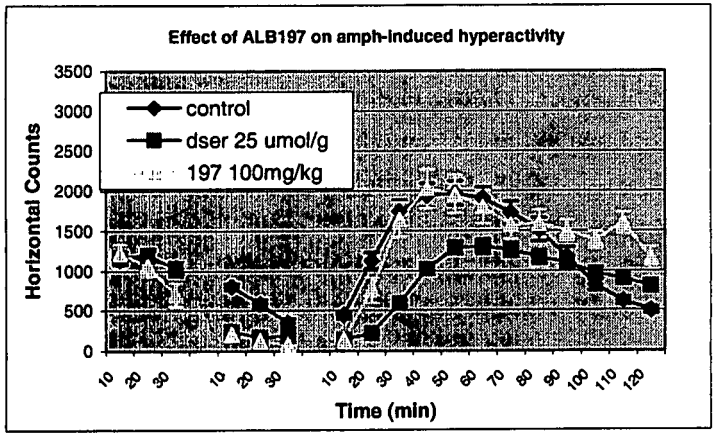
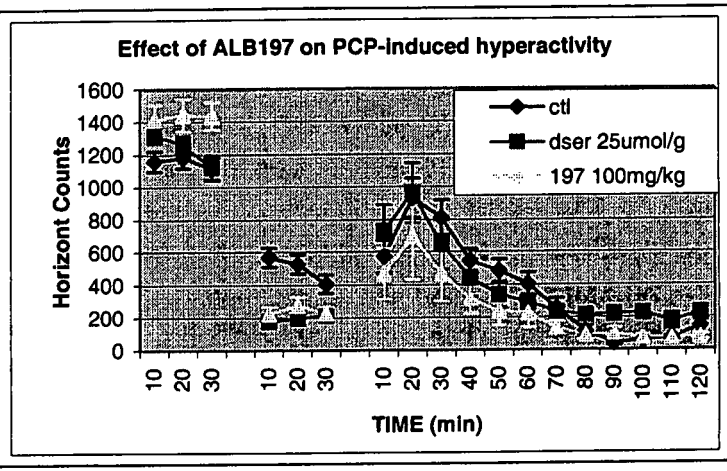
cell culture (2273 cells)			

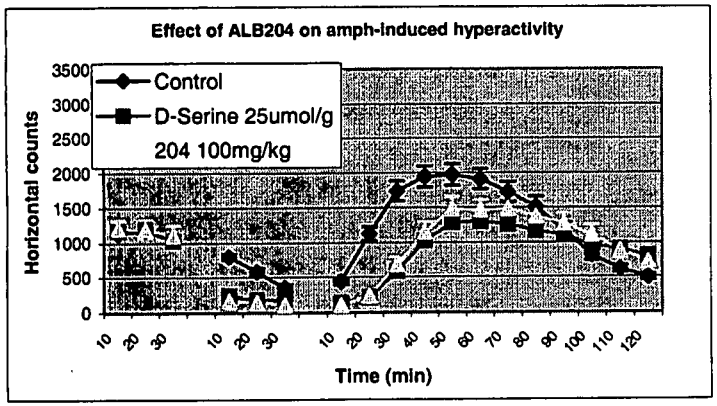
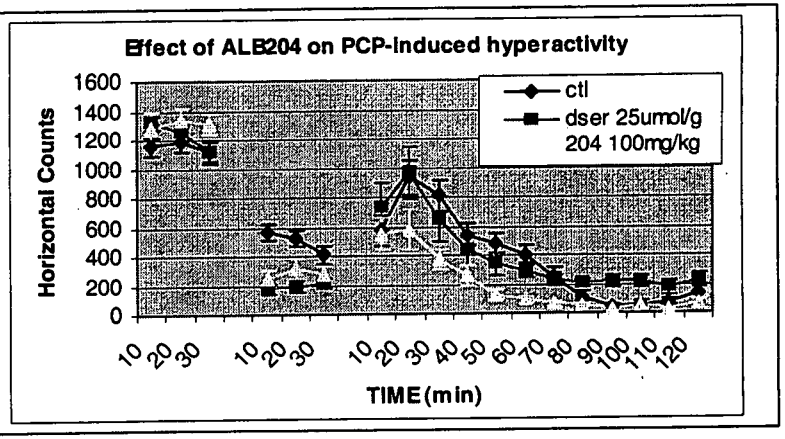
rat cortical synaptosomes		

Appendix B: Effect of Prototype D-serine **transport inhibitors on NMDA (300 µM)-induced DA release from isolated mouse striatum *in vitro***



Appendix C: Summary of animal behavior (locomotor hyperactivity) studies

	Amphetamine-induced hyperactivity	PCP-induced hyperactivity
101		
197		

204	 Effect of ALB204 on amph-induced hyperactivity Control D-Serine 25umol/g 204 100mg/kg Horizontal counts Time (min)	 Effect of ALB204 on PCP-Induced hyperactivity ctl dser 25umol/g 204 100mg/kg Horizontal Counts TIME (min)

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Daniel JAVITT

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Examiner: Shobha Kantamneni

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For: D-SERINE TRANSPORT ANTAGONIST FOR TREATING PSYCHOSIS

SUPPLEMENTAL DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Daniel Javitt, hereby declare and state:

THAT I am a citizen of the United States of America;

THAT I have received an M.D. Degree in 1983 and a Ph.D. Degree in 1990 from the Albert Einstein College of Medicine.

THAT I have been employed by the Nathan Kline Institute since 1997, where I hold a position as Psychiatrist III (research), with responsibility for Directorship of Program in Cognitive Neuroscience and Schizophrenia; and

THAT I am the inventor of the subject matter of the above-identified application and I am familiar with the proceedings in the prosecution of the present application.

Further, to the Declaration previously submitted on October 14, 2004, I provide the following as evidence that the claims are enabled by the specification as originally filed such that one of ordinary skill in the art can practice the claimed invention.

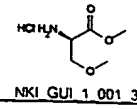
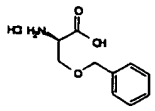
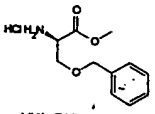
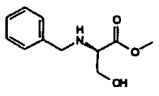
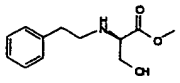
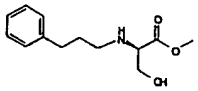
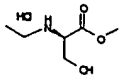
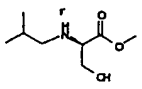
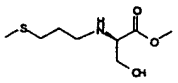
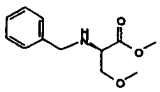
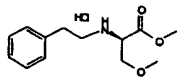
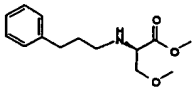
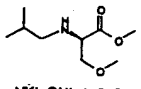
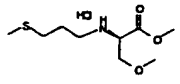
Construction of compound libraries based upon functional assays is a standard chemical approach. Since submission of the original application, using the assay described in the present specification, Applicant has obtained a library of over 200 compounds within the scope of the present claims as illustrated in the attached Appendix. Several of these compounds have shown the ability to inhibit D-Serine transport using the assay system described in the present specification. Generation of these compounds was performed by one of ordinary skill in the art, and testing was performed according to specifications provided. Thus, the specification has proven enabling to individuals skill in the art, contrary to the assertions of the Examiner.

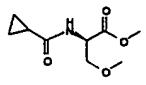
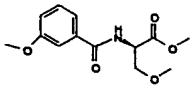
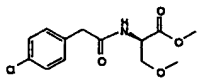
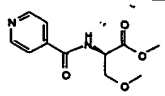
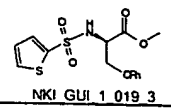
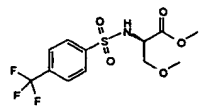
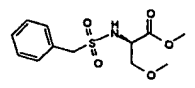
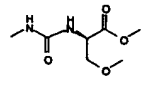
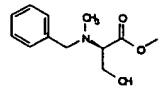
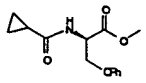
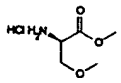
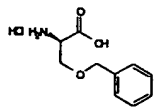
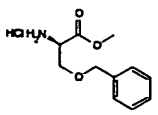
In summary, since the skilled artisan can readily determine derivative compounds of serine or alanine usable in the presently claimed method, the present claims are sufficiently enabled by the application as filed.

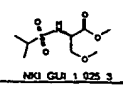
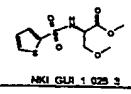
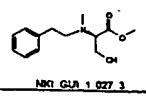
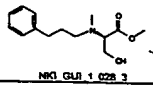
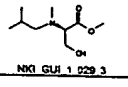
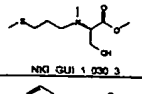
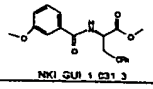
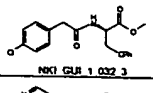
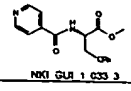
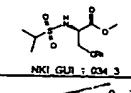
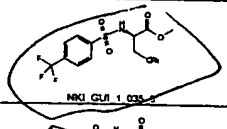
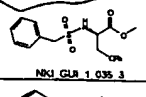
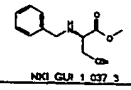
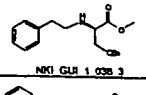
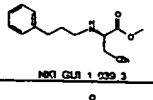
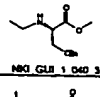
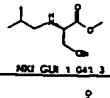
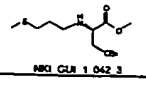
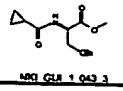
I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

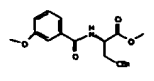
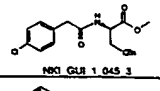
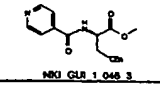
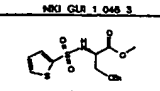
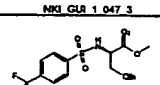
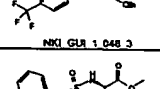
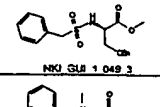
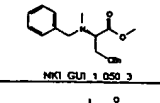
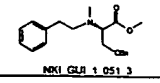
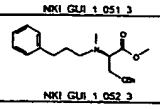
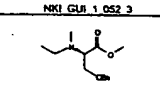
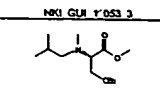
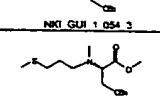
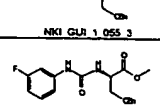
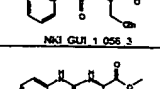
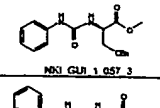
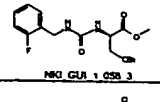
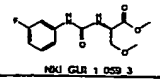
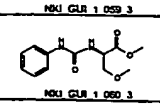
Date: 12/17/2007

Dr. Daniel Javitt

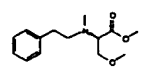
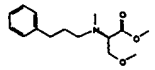
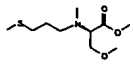
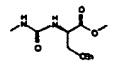
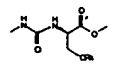
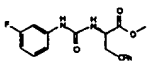
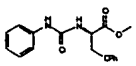
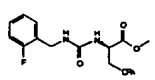
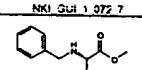
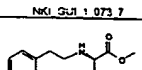
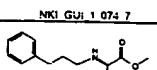
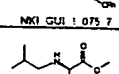
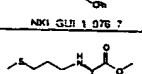
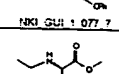
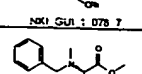
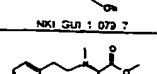
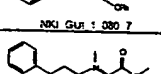
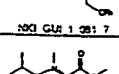
CHEMISTRY	Location	Formula	Smiles	W (g/mol)	[M+H]	HPLC purity (%)	Mass	units
 NKI_GUI_1_001_3	plate 1-A1	C5H11NO3 HCl	[C@H](COC)(N)C(=O)OC.Cl	169.61	133	>99	3	mg
 NKI_GUI_1_002_3	plate 1-A2	HCl C10H13NO3	Cl.[C@H](COCc1ccccc1)(N)C(=O)O	231.68	196	>99	3	mg
 NKI_GUI_1_003_3	plate 1-A3	HCl C11H15NO3	Cl.[C@H](COCc1ccccc1)(N)C(=O)OC	245.70	210	>99	3	mg
 NKI_GUI_1_004_3	plate 1-A4	C11H15NO3	[C@@H](CO)C(=O)OC)NCc1ccccc1	209.24	210	98	3	mg
 NKI_GUI_1_005_3	plate 1-A5	C12H17NO3	[C@H](CO)NCCc1ccccc1)C(=O)OC	223.27	224	>99	3	mg
 NKI_GUI_1_006_3	plate 1-A6	C13H19NO3	[C@H](CO)NCCCc1ccccc1)C(=O)OC	237.30	238	>99	3	mg
 NKI_GUI_1_007_3	plate 1-B1	C6H13NO3 HCl	[C@H](CO)NCC)C(=O)OC.Cl	183.63	148	98	3	mg
 NKI_GUI_1_008_3	plate 1-B2	C8H17NO3	[C@H](CO)NCC(C)C)C(=O)OC	175.23	176	92	3	mg
 NKI_GUI_1_009_3	plate 1-B3	C8H17NO3S	[C@H](CO)NCCCSC)C(=O)OC	207.29	208	90	3	mg
 NKI_GUI_1_010_3	plate 1-B4	C12H17NO3	[C@H](COC)NCc1ccccc1)C(=O)OC	223.27	224	>99	3	mg
 NKI_GUI_1_011_3	plate 1-B5	C13H19NO3 HCl	[C@H](COC)NCCc1ccccc1)C(=O)OC.Cl	273.63	238	98	3	mg
 NKI_GUI_1_012_3	plate 1-B6	C14H21NO3	[C@H](COC)NCCCc1ccccc1)C(=O)OC	251.33	252	>99	3	mg
 NKI_GUI_1_013_3	plate 1-C1	C9H19NO3	[C@H](COC)NCC(C)C)C(=O)OC	189.25	190	>99	3	mg
 NKI_GUI_1_014_3	plate 1-C2	C9H19NO3S HCl	[C@H](COC)NCCCSC)C(=O)OC.Cl	257.78	222	>99	3	mg

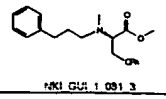
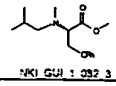
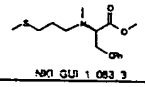
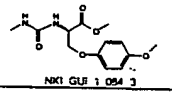
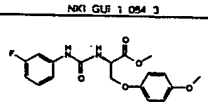
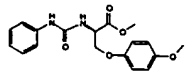
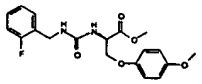
 NKI_GUI_1_015_3	plate 1-C3	C9H15NO4	[C@@H](COC)(C(=O)OC)NC(C1CC1)=O	201.22	202	>99	3	mg
 NKI_GUI_1_016_3	plate 1-C4	C13H17NO5	[C@@H](COC)(C(=O)OC)NC(c1cccc(c1)OC)=O	267.28	268	90	3	mg
 NKI_GUI_1_017_3	plate 1-C5	C13H16ClNO4	[C@@H](COC)(C(=O)OC)NC(Cc1ccc(cc1)Cl)=O	285.73	286	98	3	mg
 NKI_GUI_1_018_3	plate 1-C6	C11H14N2O4	[C@@H](COC)(C(=O)OC)NC(c1ccncc1)=O	238.24	239	>99	3	mg
 NKI_GUI_1_019_3	plate 1-D1	C14H15NO5S2	[C@@H](COc1ccccc1)(C(=O)OC)NS(c1cccs1)(=O)=O	341.40	340*	>99	3	mg
 NKI_GUI_1_020_3	plate 1-D2	C12H14F3NO5S	[C@@H](COC)(C(=O)OC)NS(c1ccc(cc1)C(F)(F)F)(=O)=O	341.30	340*	98	3	mg
 NKI_GUI_1_021_3	plate 1-D3	C12H17NO5S	[C@@H](COC)(C(=O)OC)NS(Cc1ccccc1)(=O)=O	287.33	288	>99	3	mg
 NKI_GUI_1_022_3	plate 1-D4	C7H14N2O4	[C@@H](COC)(C(=O)OC)NC(=O)NC	190.20	191	99	3	mg
 NKI_GUI_1_023_3	plate 1-D5	C12H17NO3	[C@@H](CO)(C(=O)OC)N(Cc1ccccc1)C	223.27	224	95	3	mg
 NKI_GUI_1_024_3	plate 1-D6	C14H17NO4	[C@@H](COc1ccccc1)(C(=O)OC)NC(C1CC1)=O	263.29	264	99	3	mg
			* MS of Well A19 and A20 on plate 1 were run by negative mode.					
 NKI_GUI_1_001_7	plate 2-A1	C5H11NO3 HCl	[C@H](COC)(N)C(=O)OC.Cl	169.61	133	>99	7	mg
 NKI_GUI_1_002_7	plate 2-A2	HCl C10H13NO3	Cl.[C@H](COCc1ccccc1)(N)C(=O)O	231.68	196	>99	7	mg
 NKI_GUI_1_003_7	plate 2-A3	HCl C11H15NO3	Cl.[C@H](COCc1ccccc1)(N)C(=O)OC	245.70	210	>99	7	mg

CHEMISTRY	Location	Formula	Smiles	MW (g/mol)	[M+H]	HPLC purity (%)	Mass	units
 NKI_GUI_1_025_3	plate 1-A1	C8H17NO5S	[C@@H](COC)(C(=O)OC)NS(C(C)C)(=O)=O	239.29	240	98	3	mg
 NKI_GUI_1_026_3	plate 1-A2	C9H13NO5S2	[C@@H](COC)(C(=O)OC)NS(c1cccs1)(=O)=O	279.33	278*	>99	3	mg
 NKI_GUI_1_027_3	plate 1-A3	C13H19NO3	[C@H](CO)N(CCc1ccccc1)C(C)(C)=O)OC	237.30	238	>99	3	mg
 NKI_GUI_1_028_3	plate 1-A4	C14H21NO3	[C@H](CO)N(CCCc1ccccc1)C)C(=O)OC	251.33	252	99	3	mg
 NKI_GUI_1_029_3	plate 1-A5	C9H19NO3	[C@H](CO)N(CC(C)C)C)C(=O)OC	189.25	190	>95**	3	mg
 NKI_GUI_1_030_3	plate 1-A6	C9H19NO3S	[C@H](CO)N(CCCSC)C)C(=O)OC	221.32	222	97	3	mg
 NKI_GUI_1_031_3	plate 1-B1	C16H19NO5	[C@@H](COc1ccccc1)(C(=O)OC)NC(c1cccc(c1)OC)=O	329.35	330	>99	3	mg
 NKI_GUI_1_032_3	plate 1-B2	C16H18ClNO4	[C@@H](COc1ccccc1)(C(=O)OC)NC(Cc1ccc(cc1)Cl)=O	347.80	348	>99	3	mg
 NKI_GUI_1_033_3	plate 1-B3	C16H16N2O4	[C@@H](COc1ccccc1)(C(=O)OC)NC(c1ccncc1)=O	300.31	301	>99	3	mg
 NKI_GUI_1_034_3	plate 1-B4	C13H19NO5S	[C@@H](COc1ccccc1)(C(=O)OC)NS(C(C)C)(=O)=O	301.36	300*	>99	3	mg
 NKI_GUI_1_035_3	plate 1-B5	C17H16F3NO5S	[C@@H](COc1ccccc1)(C(=O)OC)NS(c1ccc(cc1)C(F)(F)F)(=O)=O	403.37	402*	>99	3	mg
 NKI_GUI_1_036_3	plate 1-B6	C17H19NO5S	[C@@H](COc1ccccc1)(C(=O)OC)NS(Cc1ccccc1)(=O)=O	349.40	348*	>99	3	mg
 NKI_GUI_1_037_3	plate 1-C1	C18H21NO3	[C@@H](COCc1ccccc1)(C(=O)OC)NCc1ccccc1	299.37	300	>99	3	mg
 NKI_GUI_1_038_3	plate 1-C2	C19H23NO3	[C@H](COCc1ccccc1)(NCOc1ccccc1)C(=O)OC	313.40	314	>99	3	mg
 NKI_GUI_1_039_3	plate 1-C3	C20H25NO3	[C@H](COCc1ccccc1)(NCCCc1ccccc1)C(=O)OC	327.42	328	>99	3	mg
 NKI_GUI_1_040_3	plate 1-C4	C13H19NO3	[C@H](COCc1ccccc1)(NCC)C(=O)OC	237.30	238	>99	3	mg
 NKI_GUI_1_041_3	plate 1-C5	C15H23NO3	[C@H](COCc1ccccc1)(NCC(C)C)C(=O)OC	265.35	266	>99	3	mg
 NKI_GUI_1_042_3	plate 1-C6	C15H23NO3S	[C@H](COCc1ccccc1)(NCCCSC)C(=O)OC	297.42	298	>99	3	mg
 NKI_GUI_1_043_3	plate 1-D1	C13H13NO4	[C@@H](COCc1ccccc1)(C(=O)OC)NC(C1CC1)=O	277.32	278	>99	3	mg

 NKI_GUI_1_044_3	plate 1-D2	C19H21NO5	[C@@H](COCc1ccccc1)(C(=O)OC)NC(c1ccco[c1)OC)=O	343.38	344	>99	3	mg
 NKI_GUI_1_045_3	plate 1-D3	C19H20ClNO4	[C@@H](COCc1ccccc1)(C(=O)OC)NC(Oc1ccc(cc1)Cl)=O	361.82	362	>99	3	mg
 NKI_GUI_1_046_3	plate 1-D4	C17H18N2O4	[C@@H](COCc1ccccc1)(C(=O)OC)NC(c1ccncc1)=O	314.34	315	>99	3	mg
 NKI_GUI_1_047_3	plate 1-D5	C15H17NO5S2	[C@@H](COCc1ccccc1)(C(=O)OC)NS(c1cccs1)(=O)=O	355.43	356	>99	3	mg
 NKI_GUI_1_048_3	plate 1-D6	C18H18F3NO5S	[C@@H](COCc1ccccc1)(C(=O)OC)NS(c1ccc(cc1)C(F)(F)F)(=O)=O	417.40	418*	>99	3	mg
 NKI_GUI_1_049_3	plate 2-A1	C19H21NO5S	[C@@H](COCc1ccccc1)(C(=O)OC)NS(Cc1ccccc1)(=O)=O	383.43	382*	>99	3	mg
 NKI_GUI_1_050_3	plate 2-A2	C19H23NO3	[C@@H](COCc1ccccc1)(C(=O)OC)N(Cc1ccccc1)C	313.40	314	>99	3	mg
 NKI_GUI_1_051_3	plate 2-A3	C20H25NO3	[C@H](COCc1ccccc1)N(CCc1ccccc1)C)C(=O)OC	327.42	328	>99	3	mg
 NKI_GUI_1_052_3	plate 2-A4	C21H27NO3	[C@H](COCc1ccccc1)N(CCCc1ccccc1)C)C(=O)OC	341.45	342	>99	3	mg
 NKI_GUI_1_053_3	plate 2-A5	C14H21NO3	[C@H](COCc1ccccc1)N(CC)C)C(=O)OC	251.33	252	92	3	mg
 NKI_GUI_1_054_3	plate 2-A6	C16H25NO3	[C@H](COCc1ccccc1)N(CC(C)C)C)C(=O)OC	279.38	280	>99	3	mg
 NKI_GUI_1_055_3	plate 2-B1	C15H23NO3S	[C@H](COCc1ccccc1)N(CCCSC)C)C(=O)OC	311.44	312	>99	3	mg
 NKI_GUI_1_056_3	plate 2-B2	C18H19FN2O4	[C@@H](COCc1ccccc1)(C(=O)OC)NC(=O)Nc1ccc(cc1)F	348.36	347	>99	3	mg
 NKI_GUI_1_057_3	plate 2-B3	C18H20N2O4	[C@@H](COCc1ccccc1)(C(=O)OC)NC(=O)Nc1ccccc1	328.37	329	>99	3	mg
 NKI_GUI_1_058_3	plate 2-B4	C19H21FN2O4	[C@@H](COCc1ccccc1)(C(=O)OC)NC(=O)NCc1ccccc1F	360.38	361	>99	3	mg
 NKI_GUI_1_059_3	plate 2-B5	C12H15FN2O4	[C@@H](COC)(C(=O)OC)NC(=O)Nc1ccc(cc1)F	270.26	271	>99	3	mg
 NKI_GUI_1_060_3	plate 2-B6	C12H16N2O4	[C@@H](COC)(C(=O)OC)NC(=O)Nc1ccccc1	252.27	253	>99	3	mg
 NKI_GUI_1_061_3	plate 2-C1	C13H17FN2O4	[C@@H](COC)(C(=O)OC)NC(=O)NCc1ccccc1F	284.29	285	>99	3	mg
 NKI_GUI_1_062_5	plate 2-C2	C14H21NO5S	[C@@H](COCc1ccccc1)(C(=O)OC)NS(=O)(=O)C(C)C	315.39	314*	>99	5	mg

* MS of these compounds were run by negative mode. ** The purity of W all A5 on plate 1 was obtained based on ^{1}H NMR

 NKI_GUI_1_065_7	plate 3-A3	C14H21NO3	[C@H](COC)N(CCc1ccccc1)C)C(=O)OC	251.33	252	99	7	mg
 NKI_GUI_1_066_7	plate 3-A4	C15H23NO3	[C@H](COC)N(CCCc1ccccc1)C)C(=O)OC	265.35	266	99	7	mg
 NKI_GUI_1_067_7	plate 3-A5	C10H21NO3S	[C@H](COC)N(CCCSC)C)C(=O)OC	235.35	236	97	7	mg
 NKI_GUI_1_068_7	plate 3-A6	C13H18N2O4	[C@@H](COCc1ccccc1)C(=O)OC)NC(=O)NC	266.30	267	>99	7	mg
 NKI_GUI_1_069_7	plate 3-B1	C12H16N2O4	[C@@H](COc1ccccc1)C(=O)OC)NC(=O)NC	252.27	253	95	7	mg
 NKI_GUI_1_070_7	plate 3-B2	C17H17FN2O4	[C@@H](COc1ccccc1)C(=O)OC)NC(=O)Nc1cc(ccc1)F	332.33	333	>99	7	mg
 NKI_GUI_1_071_7	plate 3-B3	C17H18N2O4	[C@@H](COc1ccccc1)C(=O)OC)NC(=O)Nc1ccccc1	314.34	315	>99	7	mg
 NKI_GUI_1_072_7	plate 3-B4	C18H19FN2O4	[C@@H](COc1ccccc1)C(=O)OC)NC(=O)NCc1ccccc1F	346.36	347	>99	7	mg
 NKI_GUI_1_073_7	plate 3-B5	C17H19NO3	[C@@H](COc1ccccc1)C(=O)OC)NCc1ccccc1	285.34	286	>99	7	mg
 NKI_GUI_1_074_7	plate 3-B6	C18H21NO3	[C@H](COc1ccccc1)(NCCc1ccccc1)C(=O)OC	299.37	300	98	7	mg
 NKI_GUI_1_075_7	plate 3-C1	C19H23NO3	[C@H](COc1ccccc1)(NCCCc1ccccc1)C(=O)OC	313.40	314	>99	7	mg
 NKI_GUI_1_076_7	plate 3-C2	C14H21NO3	[C@H](COc1ccccc1)(NCC(C)C)C(=O)OC	251.33	252	>99	7	mg
 NKI_GUI_1_077_7	plate 3-C3	C14H21NO3S	[C@H](COc1ccccc1)(NCCCSC)C(=O)OC	283.39	284	98	7	mg
 NKI_GUI_1_078_7	plate 3-C4	C12H17NO3	[C@H](COc1ccccc1)(NCC)C(=O)OC	223.27	224	95	7	mg
 NKI_GUI_1_079_7	plate 3-C5	C18H21NO3	[C@@H](COc1ccccc1)C(=O)OC)N(Cc1ccccc1)C	299.37	300	>99	7	mg
 NKI_GUI_1_080_7	plate 3-C6	C19H23NO3	[C@H](COc1ccccc1)(N(CCc1ccccc1)C)C(=O)OC	313.40	314	>99	7	mg
 NKI_GUI_1_081_7	plate 3-D1	C20H25NO3	[C@H](COc1ccccc1)(N(CCCc1ccccc1)C)C(=O)OC	327.42	328	>99	7	mg
 NKI_GUI_1_082_7	plate 3-D2	C15H23NO3	[C@H](COc1ccccc1)N(CC(C)C)C)C(=O)OC	265.35	266	97	7	mg

 NKI GUI 1 081 3	plate 1-D1	C20H25NO3	[C@H](COc1ccccc1)(N(CCCc1ccccc1)C)C(=O)OC	327.42	328	>99	3	mg
 NKI GUI 1 082 3	plate 1-D2	C15H23NO3	[C@H](COc1ccccc1)(N(CC(C)C)C)C(=O)OC	265.35	266	97	3	mg
 NKI GUI 1 083 3	plate 1-D3	C15H23NO3S	[C@H](COc1ccccc1)(N(CCCSC)C)C(=O)OC	297.42	298	95	3	mg
 NKI GUI 1 084 3	plate 1-D4	C13H18N2O5	[C@@H](COc1ccc(cc1)OC)(C(=O)OC)NC(=O)NC	282.30	283	>99	3	mg
 NKI GUI 1 085 3	plate 1-D5	C18H19FN2O5	[C@@H](COc1ccc(cc1)OC)(C(=O)OC)NC(=O)Nc1cc(ccc1)F	362.36	363	>99	3	mg
 NKI GUI 1 086 3	plate 1-D6	C18H20N2O5	[C@@H](COc1ccc(cc1)OC)(C(=O)OC)NC(=O)Nc1ccccc1	344.37	345	>99	3	mg
 NKI GUI 1 087 3	plate 2-A1	C19H21FN2O5	[C@@H](COc1ccc(cc1)OC)(C(=O)OC)NC(=O)NCc1ccccc1F	376.38	377	>99	3	mg
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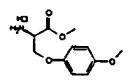
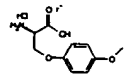
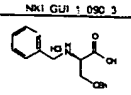
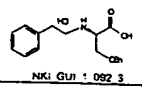
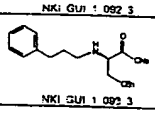
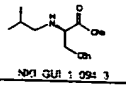
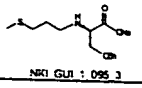
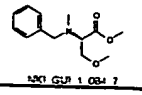
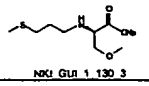
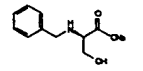
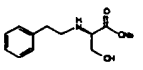
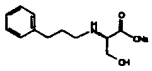
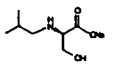
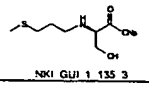
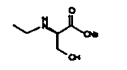
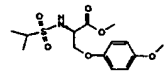
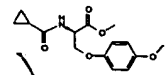
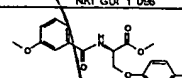
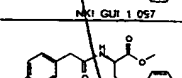
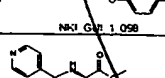
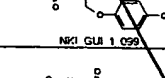
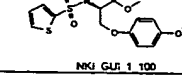
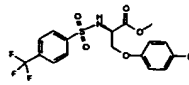
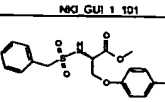
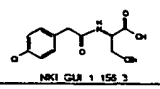
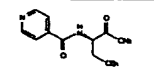
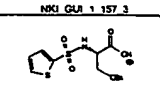
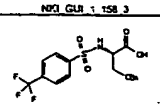
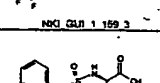
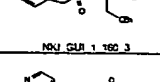
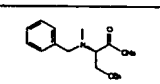
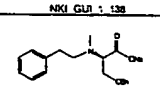
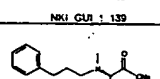
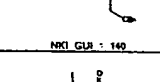
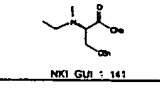
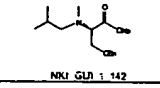
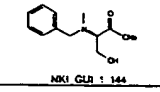
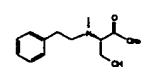
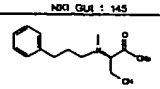
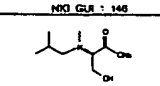
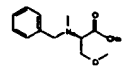
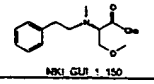
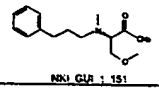
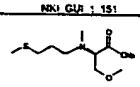
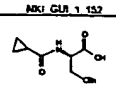
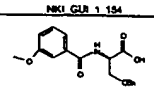
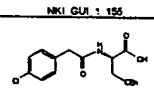
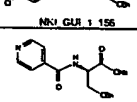
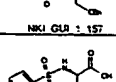
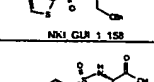
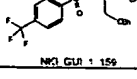
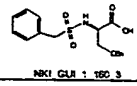
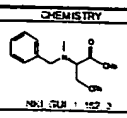
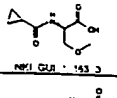
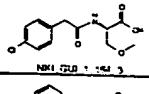
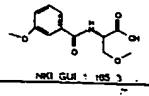
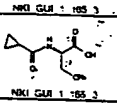
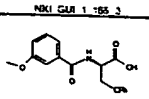
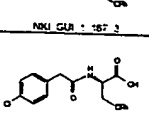
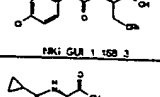
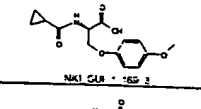
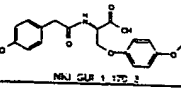
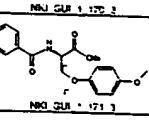
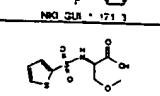
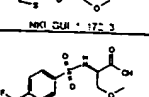
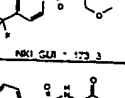
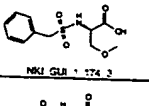
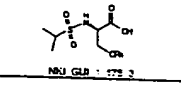
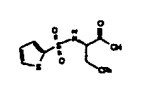
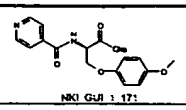
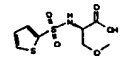
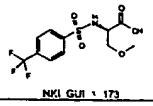
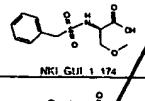
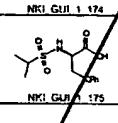
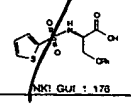
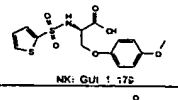
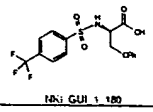
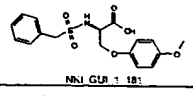
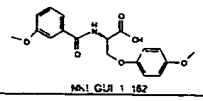
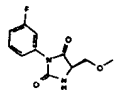
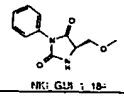
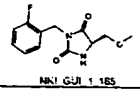
 NKI GUI 1 089 3	plate 2-A3	C11H15NO4 HCl	[C@@H](COc1ccc(cc1)OC)(C(=O)O)N.Cl	261.70	226	>99	3	mg
 NKI GUI 1 090 3	plate 2-A4	C10H13NO4 HCl	[C@@H](COc1ccc(cc1)OC)(C(=O)O)N.Cl	247.68	212	94	3	mg
 NKI GUI 1 091 3	plate 2-A5	C17H19NO3 HCl	[C@@H](COCc1ccccc1)(C(=O)O)NCc1ccccc1.Cl	321.80	286	>99	3	mg
 NKI GUI 1 092 3	plate 2-A6	C18H21NO3 HCl	[C@H](COCc1ccccc1)(NCCc1ccccc1)C(=O)O.Cl	335.83	300	>99	3	mg
 NKI GUI 1 093 3	plate 2-B1	C19H22NNaO3	[C@H](COCc1ccccc1)(NCCCc1ccccc1)C(=O)O[Na]	335.38	313	98	3	mg
 NKI GUI 1 094 3	plate 2-B2	C14H20NNaO3	[C@H](COCc1ccccc1)(NCC(C)C)C(=O)O[Na]	273.31	251	98	3	mg
 NKI GUI 1 095 3	plate 2-B3	C14H20NNaO3S	[C@H](COCc1ccccc1)(NCCCSC)C(=O)O[Na]	305.37	284	94	3	mg
CHEMISTRY	Location	Formula	Smiles	MW (g/mol)	[M+H]	HPLC purity (%)	Mass	units
 NKI GUI 1 083 7	plate 3-A1	C10H13NO3 HCl	[C@@H](COc1ccccc1)(C(=O)OC)N.Cl	231.68	196	>99	7	mg
 NKI GUI 1 084 7	plate 3-A2	C13H19NO3	[C@@H](COC)(C(=O)OC)NCc1ccccc1)C	237.30	238	>99	7	mg

 NKI_GUI_1_130_3	plate 2-B5	C8H16NNaO3S	[C@H](COC)(NCCCSC)C(=O)O[Na]	229.27	208	95	3	mg
 NKI_GUI_1_131_3	plate 2-B6	C10H12NNaO3	[C@@H](CO)(C(=O)O[Na])NCc1ccccc1	217.20	196	95	3	mg
 NKI_GUI_1_132_3	plate 2-C1	C11H14NNaO3	[C@H](CO)(NCCc1ccccc1)C(=O)O[Na]	231.23	210	98	3	mg
 NKI_GUI_1_133_3	plate 2-C2	C12H15NNaO3	[C@H](CO)(NCCCc1ccccc1)C(=O)O[Na]	245.25	224	98	3	mg
 NKI_GUI_1_134_3	plate 2-C3	C7H14NNaO3	[C@H](CO)(NCC(C)C)C(=O)O[Na]	183.18	162	>99	3	mg
 NKI_GUI_1_135_3	plate 2-C4	C7H14NNaO3S	[C@H](CO)(NCCCSC)C(=O)O[Na]	215.25	194	98	3	mg
 NKI_GUI_1_136_3	plate 2-C5	C5H10NNaO3	[C@H](CO)(NCC)C(=O)O[Na]	155.13	134	>99	3	mg
 NKI_GUI_1_137_3	plate 2-C6	C14H21NO6S	[C@@H](COc1ccc(cc1)OC)(C(=O)OC)NS(=O)(=O)C(C)C	331.39	330*	>99	3	mg
			* MS of these compounds were run by negative mode.					
CHEMISTRY	Location	Formula	Smiles	MW (g/mol)	[M+H]	HPLC purity (%)	Mass	mg
 NKI_GUI_1_096	plate 3-A1	C15H19NO5	[C@@H](COc1ccc(cc1)OC)(C(=O)OC)NC(=O)C1CC1	293.32	294	98	7	mg
 NKI_GUI_1_097	plate 3-A2	C19H21NO6	[C@@H](COc1ccc(cc1)OC)(C(=O)OC)NC(=O)c1cccc(c1)OC	359.38	360	>99	7	mg
 NKI_GUI_1_098	plate 3-A3	C19H20ClNO5	[C@@H](COc1ccc(cc1)OC)(C(=O)OC)NC(Cc1ccc(cc1)Cl)=O	377.82	378	>99	7	mg
 NKI_GUI_1_099	plate 3-A4	C17H18N2O5	[C@@H](COc1ccc(cc1)OC)(C(=O)OC)NC(=O)c1ccncc1	330.34	331	>99	7	mg
 NKI_GUI_1_100	plate 3-A5	C15H17NO6S2	[C@@H](COc1ccc(cc1)OC)(C(=O)OC)NS(c1cccs1)(=O)=O	371.43	370*	>99	7	mg
 NKI_GUI_1_101	plate 3-A6	C18H18F3NO6S	[C@@H](COc1ccc(cc1)OC)(C(=O)OC)NS(c1ccc(cc1)C(F)(F)F)(=O)=O	433.40	432*	>99	7	mg
 NKI_GUI_1_102	plate 3-B1	C18H21NO6S	[C@@H](COc1ccc(cc1)OC)(C(=O)OC)NS(Cc1ccccc1)(=O)=O	379.43	378*	>99	7	mg
 NKI_GUI_1_103	plate 3-B2	C18H21NO4	[C@@H](COc1ccc(cc1)OC)(C(=O)OC)NCc1ccccc1	315.37	316	>99	7	mg

 NKI GUI 1 156 3	plate 1-D1	C18H18ClNO4	[C@@H]([COCc1ccccc1)(C(=O)O)NC(Cc1ccc(cc1)Cl)=O	347.80	348*	98	3	mg
 NKI GUI 1 157 3	plate 1-D2	C16H15N2Na O4	[C@@H]([COCc1ccccc1)(C(=O)O)O[Na])NC(c1ccncc1)=O	322.30	301	92	3	mg
 NKI GUI 1 158 3	plate 1-D3	C14H15NO5S2	[C@@H]([COCc1ccccc1)(C(=O)O)NS(c1cccs1)(=O)=O	341.40	340*	>99	3	mg
 NKI GUI 1 159 3	plate 1-D4	C17H16F3NO5S	[C@@H]([COCc1ccccc1)(C(=O)O)NS(c1ccc(cc1)C(F)(F)F)(=O)=O	403.37	402*	>99	3	mg
 NKI GUI 1 160 3	plate 1-D5	C17H19NO5S	[C@@H]([COCc1ccccc1)(C(=O)O)NS(=O)(=O)Cc1ccccc1	349.40	348*	>99	3	mg
 NKI GUI 1 161 3	plate 1-D6	C10H11N2Na O4	[C@@H]([COC)(C(=O)O)O[Na])NC(c1ccncc1)=O	246.20	225	>99	3	mg
			* MS of these compounds were run by negative mode. ** The purity of Well B5 on plate 1 was obtained based on ^{1}H NMR.					
 NKI GUI 1 138	plate 2-A1	C18H20NNa O3	[C@@H]([COCc1ccccc1)(C(=O)O)O[Na])N(Cc1ccccc1)C	321.35	300	99	49	mg
 NKI GUI 1 139	plate 2-A2	C19H22NNa O3	[C@H]([COCc1ccccc1)N(CCc1ccccc1)C)C(=O)O[Na]	335.38	314	>99	19	mg
 NKI GUI 1 140	plate 2-A3	C20H24NNa O3	[C@H]([COCc1ccccc1)N(CCCc1ccccc1)C)C(=O)O[Na]	349.41	328	>99	83	mg
 NKI GUI 1 141	plate 2-A4	C13H18NNa O3	[C@H]([COCc1ccccc1)N(CC)C)C(=O)O[Na]	259.28	238*	>99	16	mg
 NKI GUI 1 142	plate 2-A5	C15H22NNa O3	[C@H]([COCc1ccccc1)N(CC(C)C)C)C(=O)O[Na]	287.33	266	>99	37	mg
 NKI GUI 1 144	plate 2-B1	C11H14NNa O3	[C@@H](CO)(C(=O)O[Na])N(Cc1ccccc1)C	231.23	210	90	33	mg
 NKI GUI 1 145	plate 2-B2	C12H16NNa O3	[C@H]([CO)N(CCc1ccccc1)C)C(=O)O[Na]	245.25	224	94	37	mg
 NKI GUI 1 146	plate 2-B3	C13H18NNa O3	[C@H]([CO)N(CCCc1ccccc1)C)C(=O)O[Na]	259.28	238	98	20	mg
 NKI GUI 1 147	plate 2-B4	C8H15NNa O3	[C@H]([CO)N(CC(C)C)C)C(=O)O[Na]	197.21	176	98	27	mg
 NKI GUI 1 148	plate 2-B5	C8H15NNa O3S	[C@H]([CO)N(CCCSC)C)C(=O)O[Na]	229.27	208	>95**	39	mg

 NKI_GUI_1_149	plate 2-B6	C12H16NNa O3	[C@@H](COC)(C(=O)O[Na])N(Cc1ccccc1)C	245.25	224	95	8	mg
 NKI_GUI_1_150	plate 2-C1	C13H18NNa O3	[C@H](COC)N(CCc1ccccc1)C(=O)O[Na]	259.28	238	>99	13	mg
 NKI_GUI_1_151	plate 2-C2	C14H20NNa O3	[C@H](COC)N(CCCc1ccccc1)C(C(=O)O[Na]	273.31	252	>99	29	mg
 NKI_GUI_1_152	plate 2-C3	C8H16NNa O3S	[C@H](COC)N(CCCSC)C(C(=O)O[Na]	243.30	222	90	27	mg
 NKI_GUI_1_154	plate 2-C5	C14H17NO4	[C@@H](COCc1ccccc1)(C(=O)O)NC(C1CC1)=O	263.29	262*	98	44	mg
 NKI_GUI_1_155	plate 2-C6	C18H19NO6	[C@@H](COCc1ccccc1)(C(=O)O)NC(c1cccc(c1)OC)=O	329.35	328*	98	58	mg
 NKI_GUI_1_156	plate 2-D1	C18H18ClNO4	[C@@H](COCc1ccccc1)(C(=O)O)NC(Cc1ccc(cc1)Cl)=O	347.80	346*	98	43	mg
 NKI_GUI_1_157	plate 2-D2	C15H15N2Na O4	[C@@H](.COCc1ccccc1)(C(=O)O)[Na]NC(c1ccncc1)=O	322.30	301	92	51	mg
 NKI_GUI_1_158	plate 2-D3	C14H15NO5S2	[C@@H](COCc1ccccc1)(C(=O)O)NS(c1cccs1)(=O)=O	341.40	340*	>99	52	mg
 NKI_GUI_1_159	plate 2-D4	C17H16F3NO5S	[C@@H](COCc1ccccc1)(C(=O)O)NS(c1ccc(cc1)C(F)(F)F)(=O)=O	403.37	402*	>99	40	mg
 NKI_GUI_1_160_3	plate 2-D5	C17H19NO5S	[C@@H](COCc1ccccc1)(C(=O)O)NS(=O)(=O)Cc1ccccc1	349.40	348*	>99	20	mg
 NKI_GUI_1_161	plate 2-D6	C10H11N2Na O4	[C@@H](COC)(C(=O)O)[Na]NC(c1ccncc1)=O	246.20	225	>99	32	mg
			* MS of these compounds were run by negative mode. ** The purity of Well B5 on plate 2 was obtained based on 1H NMR.					

CHEMISTRY	Location	Formula	SMILES	MW (g/mol)	[M+H]+	HPLC purity (%)	Mass	mg
 NKI_GUI_1_167_3	plate 1-A1	C17H18BrNa O3	[C@@H](CC(=O)O)N(C)C(=O)OCc1ccccc1)c1ccc(Br)cc1	307.32	286*	94	3	mg
 NKI_GUI_1_163_3	plate 1-A2	C8H13NO4	[C@@H](C(=O)O)N(C)C(=O)C1CC1	187.19	186	>99	3	mg
 NKI_GUI_1_164_3	plate 1-A3	C12H14ClNO4	[C@@H](CC(=O)O)N(C)C(=O)Cc1ccccc1Cl	271.70	270	>99	3	mg
 NKI_GUI_1_165_3	plate 1-A4	C12H15NO5	[C@@H](CC(=O)O)N(C)C(=O)c1ccccc1OC	253.25	252	>99	3	mg
 NKI_GUI_1_166_3	plate 1-A5	C13H15NO4	[C@@H](CC(=O)O)N(C)C(=O)Cc1ccccc1	249.27	248	98	3	mg
 NKI_GUI_1_167_3	plate 1-A6	C17H17NO5	[C@@H](CC(=O)O)N(C)C(=O)c1ccccc1OCc1ccccc1	315.32	314	89	3	mg
 NKI_GUI_1_168_3	plate 1-B1	C17H18ClNO4	[C@@H](CC(=O)O)N(C)C(=O)Cc1ccccc1Cl	333.77	332	92	3	mg
 NKI_GUI_1_169_3	plate 1-B2	C14H17NO5	[C@@H](CC(=O)O)N(C)C(=O)COc1ccc(OC)cc1	279.29	278	98	3	mg
 NKI_GUI_1_170_3	plate 1-B3	C18H18ClNO5	[C@@H](CC(=O)O)N(C)C(=O)COc1ccc(OC)c(Cl)c1	363.80	362	91	3	mg
 NKI_GUI_1_171_3	plate 1-B4	C16H15N2Na O5	[C@@H](CC(=O)O)N(C)C(=O)COc1ccc(cc1)C(=O)Na	338.29	317*	>95**	3	mg
 NKI_GUI_1_172_3	plate 1-B5	C8H11NO5S2	[C@@H](CO)C(=O)O)NS(=O)(=O)c1ccco1	265.31	264	99	3	mg
 NKI_GUI_1_173_3	plate 1-B6	C11H12F3NO5S	[C@@H](CO)C(=O)O)NS(=O)(=O)c1ccc(cc1)C(F)(F)F	327.28	326	99	3	mg
 NKI_GUI_1_174_3	plate 1-C1	C11H15NO5S	[C@@H](CO)C(=O)O)NS(=O)(=O)Cc1ccccc1	273.31	272	93	3	mg
 NKI_GUI_1_175_3	plate 1-C2	C12H17NO5S	[C@@H](CC(=O)O)N(C)C(=O)NS(C(C)C)(=O)=O	287.33	286	>99	3	mg
 NKI_GUI_1_176_3	plate 1-C3	C13H13NO5S2	[C@@H](CC(=O)O)N(C)C(=O)NS(=O)(=O)c1ccccc1	327.38	326	98	3	mg
 NKI_GUI_1_177_3	plate 1-C4	C15H14F3NO5S	[C@@H](CC(=O)O)N(C)C(=O)NS(=O)(=O)c1ccc(cc1)C(F)(F)F	369.35	368	86	3	mg
 NKI_GUI_1_178_3	plate 1-C5	C15H17NO5S	[C@@H](CC(=O)O)N(C)C(=O)NS(=O)(=O)c1ccccc1	335.38	334	98	3	mg

 NKI GUI 1 171	plate 2-B4	C16H16N2Na O5	[C@@H](COc1ccc(cc1)OC)(C(=O)O[Na])NC(c1ccncc1)=O	338.29	317*	>95**	7	mg
 NKI GUI 1 172	plate 2-B5	C8H11NO5S2	[C@@H](COC)(C(=O)O)NS(c1cccs1)(=O)=O	265.31	264	99	40	mg
 NKI GUI 1 173	plate 2-B6	C11H12F3NO5S	[C@@H](COC)(C(=O)O)NS(c1ccc(cc1)C(F)(F)F)(=O)=O	327.28	326	99	52	mg
 NKI GUI 1 174	plate 2-C	C11H15NO5S	[C@@H](COC)(C(=O)O)NS(Cc1ccccc1)(=O)=O	273.31	272	93	10	mg
 NKI GUI 1 175	plate 2-C2	C12H17NO5S	[C@@H](COc1ccccc1)(C(=O)O)NS(C(C)C)(=O)=O	287.33	286	>99	2	mg
 NKI GUI 1 176	plate 2-C3	C13H13NO5S2	[C@@H](COc1ccccc1)(C(=O)O)NS(c1cccs1)(=O)=O	327.38	326	98	35	mg
 NKI GUI 1 177	plate 2-C8	C14H15NO6S2	[C@@H](COc1ccc(cc1)OC)(C(=O)O)NS(c1cccs1)(=O)=O	357.40	356	96	39	mg
 NKI GUI 1 180	plate 2-D1	C17H16F3NO6S	[C@@H](COc1ccc(cc1)OC)(C(=O)O)NS(c1ccc(cc1)C(F)(F)F)(=O)=O	419.37	418	86	36	mg
 NKI GUI 1 181	plate 2-D2	C17H19NO6S	[C@@H](COc1ccc(cc1)OC)(C(=O)O)NS(Cc1ccccc1)(=O)=O	365.40	364	96	20	mg
 NKI GUI 1 182	plate 2-D3	C18H19NO6	[C@@H](COc1ccc(cc1)OC)(C(=O)O)NC(c1ccccc1)OC)=O	345.35	344	98	4	mg
 NKI GUI 1 183	plate 2-D4	C11H11FN2O3	N1[C@H](N[C@@H](C1=O)COC)=O)c1ccc(cc1)F	238.22	237	97	44	mg
 NKI GUI 1 184	plate 2-D5	C11H12N2O3	N1[C@H](N[C@@H](C1=O)COC)=O)c1ccccc1	220.23	219	>99	40	mg
 NKI GUI 1 185	plate 2-D6	C12H13FN2O3	N1[C@H](N[C@@H](C1=O)COC)=O)Cc1ccccc1)F	252.24	251	>99	13	mg
			* MS of these compounds were run in positive mode. ** The purity of Well B4 on plate 2 was obtained based on ^{1}H NMR.					

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Structural Requirements for Activation of the Glycine Coagonist Site of *N*-Methyl-D-aspartate Receptors Expressed in *Xenopus* Oocytes

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SUMMARY

Five structural features important for activation of the glycine recognition site on *N*-methyl-D-aspartate (NMDA) receptors were identified by structure-activity studies of more than 60 glycine analogues in voltage-clamped *Xenopus* oocytes injected with rat brain mRNA. First, sterically unhindered and ionized carboxyl and amino termini were essential for action at this site. Second, an increase in the interterminal separation by greater than one carbon (e.g., β-alanine) markedly attenuated activity at this site. Third, activity at the glycine site was stereoselective. The D-isomers of alanine and serine were ~20 and 30 times more potent than the L-isomers. Fourth, only small sterically unobtrusive substitutions at the α-carbon could be tolerated. α-Methyl (D-alanine) and α-cyclopropyl (1-amino-cyclopropane carboxylic acid) (ACC) substitutions were effective as agonists but most larger aliphatic and aromatic α-carbon substitutions were simply inactive. Glycine, D-alanine, and ACC probably have only a two-point attachment to the receptor. Finally the α-carbon substituent of D-serine is envisioned as binding to a third site on the receptor probably via hydrogen bonding of the ω-terminal hydroxyl group. Thus, serine, an hydroxymethyl substitution of glycine, permitted activation of NMDA receptor-mediated currents, whereas isosteric substitutions incapable of hydrogen bonding (e.g., 2-aminobutyric acid) were inactive. Additionally, the position and size of the hydroxyl-containing group is critical for agonist action; D-threonine, DL-homoserine, and hydroxyphenolic substitutions at the α-carbon were all inactive. Halogenated analogs of a size comparable to D-serine but capable only of proton acceptance at the ω-terminus (β-fluoro-D-alanine and β-chloro-D-alanine) possessed agonist action, whereas an analog capable of only proton donation (1,2-diaminopropionic acid) was inactive. Full concentration-response curves were constructed for those analogs displaying >25% of the effect of glycine when tested at 3 μM. With the exception of (*R*)-(+)cycloserine and β-fluoro-D-alanine, all compounds were nearly full agonists and had Hill coefficients not significantly different from unity. The order of relative potency of the active analogs was ACC > glycine > D-serine > D-alanine > β-fluoro-D-alanine > (*R*)-(+)-cycloserine > L-serine > L-alanine. Molecular modelling of a series of active and inactive analogs with close structural relation to glycine was undertaken. These results were complementary to those data obtained from the electrophysiological investigation. Taken together, electrophysiological and modelling data offer compelling evidence that the active site of the glycine recognition site is a small pocket containing at least three points of possible attachment, negative and positive ionic sites and an hydrogen bond-donating site. Thus, the glycine recognition site shows many of the characteristics observed for the NMDA recognition site.

Responses to the excitatory amino acid NMDA are markedly augmented by glycine, through an action at strychnine-insensitive binding sites. The exact mechanisms of glycine potentiation are unclear at present. Glycine has been demonstrated, however, to increase the frequency of NMDA receptor-associated cation channel opening without altering the mean open time or conductance in outside-out patches of murine cultured neurones (1). Recently, Mayer *et al.* (2) demonstrated that one role of glycine is to increase the rate of recovery of the NMDA receptor from the desensitized state. It is thought that glycine binds to a single discrete site on central neurones with an affinity constant of ~300 nM (3) and that the glycine sites colocalize with NMDA-preferring L-[^{3}H]glutamate binding sites in rat brain (4, 5) and L-[^{3}H]glutamate and [^{3}H]TCP binding sites in human hippocampus (6). In addition, glycine has been shown to potentiate, in a strychnine-insensitive manner, the NMDA-induced binding of noncompetitive blockers of the NMDA receptor channel (7, 8) and to potentiate preferentially NMDA receptor agonist binding in the thalamus and

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; MBS, modified Barth's solution; ACC, 1-aminocyclopropane-1-carboxylic acid; HEPES, 4-(2-hydroxyphenyl)-*N*-(1-propyl)piperidine; PCP, phencyclidine; RMS, root mean square; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; TCP, 1-(1-(2-thienyl)-cyclohexyl-piperdine.

cerebral cortex in rat brain (9). Glycine and Zn^{2+} are also known to modulate synaptic transmission in cultured mouse hippocampal neurones (10) and glycine antagonists suppress burst firing in hippocampal slices induced by perfusion with low Mg^{2+} concentration-containing solutions (11). Thus, the NMDA receptor complex has been considered as being endowed with both positive (glycine) and negative (PCP, Mg^{2+}, and Zn^{2+}) modulatory sites.

Kleckner and Dingledine (12) have shown in mRNA-injected *Xenopus* oocytes that the presence of glycine was required for measurable NMDA receptor activation, a finding recently supported in studies of central neurones (13). In *Xenopus* oocytes, glycine activation has a Hill coefficient equal to unity, with an apparent affinity constant close to that reported for mammalian central neurones. Results to date indicate that the NMDA receptor complex expressed in oocytes possesses properties virtually identical to neuronal NMDA receptors. *Xenopus* oocytes, therefore, offer a novel preparation that is well suited for electrophysiological structure-activity bioassay, without the complications normally associated with neuronal preparations, e.g., potential release of glycine or modulators from surrounding neurones/glial cells, drug diffusion barriers, and uptake.

It has been postulated that the glycine binding site on the NMDA receptor may have originally arisen from modification of the NMDA binding site (12). Structure-activity studies have highlighted that agonists bind at the NMDA receptor through a "three-point" attachment (14). Several studies have demonstrated that a small number of amino acids with structural similarity to glycine may substitute for glycine in activating NMDA receptors in both electrophysiological (1, 12) and radioligand binding (15–18) studies. A detailed examination of structure-activity relationships for activation of this glycine receptor has as yet, however, not been undertaken. We have investigated the structure-activity relationship of over 60 analogs of glycine for their ability to permit currents activated by NMDA in mRNA-injected *Xenopus* oocytes held under voltage clamp. We have determined that both carboxyl and amino termini must be left unhindered. Only small substitutions on the α-carbon, preferentially in the D-configuration, are permissable. Our data suggest that glycine has a two-point attachment through its carboxyl and amino termini to its binding site. D-Serine and structurally related compounds, however, probably owe their efficacy to additional hydrogen bonding via the ω-terminal to a third binding site within the receptive field. Comparison with NMDA receptor structure-activity studies suggests that the glycine and NMDA recognition site share many similar structural requirements for agonist activity and we propose that they may share a common ancestry.

Materials and Methods

Purification of rat brain RNA. RNA was extracted from Sprague Dawley rat brain using the technique of Chirgwin *et al.* (19), as described in detail earlier (20). The poly(A)$^+$ RNA was selected with one round of oligo(dT)cellulose chromatography (21). The isolated RNA preparations were dissolved in sterile distilled water, at a final concentration of 0.8–1.2 mg of RNA/ml, and stored in single-use aliquots at −70°.

RNA injection of *Xenopus* oocytes. RNA injection of *Xenopus* oocytes was performed as described previously (20). Briefly, *Xenopus laevis* females (Xenopus I, Ann Arbor, MI) were anaesthetized with 0.12–0.16% tricaine methylsulfonate. A small incision was made in the abdomen and lobes of ovary were removed to the culture solution, MBS (in mM, NaCl, 88; KCl, 1; $NaHCO_3$, 2.4; HEPES, 10; $MgSO_4$, 0.82; $Ca(NO_3)_2$, 0.33; $CaCl_2$, 0.91, and supplemented with penicillin and streptomycin, 1000 units/ml). The abdominal musculature was sutured and allowed to heal for a period of at least 3 weeks before further surgery was performed. The lobes of the ovary were sectioned into small pieces and shaken gently in neutral protease (1.5 mg/ml) in MBS for approximately 1 hr, until oocytes began to dissociate from the ovaries. In order to reduce diffusion barriers and to prevent possible contamination by endogenously released glycine or other modulators, oocytes were defolliculated using fine jewelers forceps after shrinking in hypertonic solution (MBS containing 100 mM sucrose). Defolliculated oocytes were then injected with ~50 nl (50 ng) of poly(A)$^+$ RNA and individually cultured at 19° for at least 48 hr before use. Oocytes typically survived 5 days following removal from the animal.

Voltage clamp of injected oocytes. Since glycine is an ubiquitous compound, all glassware was baked at 280° for 5 hr to remove glycine. The water used to prepare the perfusion medium was distilled in a closed system using baked glassware. Oocytes were positioned in a small recording chamber (volume, ~500 μl) and superfused with MBS with a composition identical to that used for culturing, with the exception that $MgSO_4$ was replaced with $NaSO_4$ (0.55 mM), an additional 0.5 mM $CaCl_2$ was added, and no antibiotics were included. Oocytes were impaled, under visual control, with glass microelectrodes filled with $CsCl_2$ and EGTA (3 M and 100 mM, respectively); electrodes had DC resistances of 1.0–5.0 MΩ when measured in physiological saline. Oocytes with input resistances of >0.5 MΩ measured at resting potential were voltage clamped with one or two microelectrodes (Axoclamp 2A; Axon Instruments) at a holding potential of −60 or −70 mV. Oocytes were perfused by gravity feed at a rate of 3.5–4.0 ml/min at room temperature. Drugs were directly dissolved in the perfusate and applied in known concentrations by way of a three-way tap assembly, with a dead time of ~6 sec. Solution changes were usually completed within 40 sec. Current and voltage signals were recorded on FM tape and chart recorder. The current signal was low-pass filtered with an eight-pole Bessel filter at 15 Hz and digitized by an IBM-AT computer (30 Hz) for later analysis.

Each glycine analog was applied to the oocyte by perfusion at a single concentration (3 μM) for a 5-min period. Following this initial incubation, the perfusate was changed to one containing both the glycine analog (3 μM) and NMDA (100 μM). The effectiveness of each analog was assessed initially as a percentage of the maximum steady state current evoked by NMDA (100 μM) and glycine (3 μM) in that particular oocyte. Full concentration-response curves were then constructed for those agonists that had >25% effectiveness, compared with that of glycine. Concentration-response curves were obtained in a fixed NMDA concentration (100 μM) by applying sequentially increasing concentrations of the glycine analog for a time sufficient to produce a maximum current at each concentration (Fig. 1). The analog-containing solution was then removed and a period of at least 3 min was allowed to elapse before the next solution was applied. The percentage analog-induced peak current was plotted against analog concentration and the curve was fitted by a nonlinear least squares program to the logistic equation

$$\text{Current} = \text{maximum current}/[1 + (EC_{50}/(\text{agonist})^n)]$$

where n and EC_{50} represent the Hill coefficient and the agonist concentration that elicited a half-maximal response, respectively.

Methods for molecular modelling. All molecular structures were either constructed using the interactive graphics program MODEL, version 2.93, or were retrieved from the MODEL template file and inverted to afford the D-configuration. Molecular mechanics-based energy minimization was performed on each analog to obtain a low energy conformation, as a starting point for a systematic conformational space search using SYBYL 5.10. Starting geometries were reminimized in SYBYL 5.10 using Maximin2. In order to search conformational space to obtain the lowest energy conformation, each resulting starting structure was subjected to systematic conformational search

in SYBYL by driving the torsion angles associated with the carboxyl carbon to α-carbon bond, α-carbon to β-carbon bond, and β-carbon to β-substituent bond. Each angle was rotated in 10° increments through 360°, while a single-point energy calculation was performed after each increment. As a search constraint, all conformations were eliminated whose energy values were more than 0.1 kcal/mol greater than the starting energy. Because the analogs are completely ionized at pH 7.4, the carboxyl proton was removed and the nitrogen protonated before the minimization and subsequent search, in order to allow for realistic steric arrangement. During the search, 46,656 conformations were examined for each analog. Upon completion of the conformational search, each lowest energy structure was reminimized with Maximin2 to allow the molecular geometry to relax. The resulting conformations were taken as the most stable for each analog. All graphics were visualized in SYBYL using an Evans and Sutherland PS390 terminal.

RMS fits were performed for each of the above lowest energy conformations in SYBYL, comparing the fit of the two equivalent carboxyl oxygens, the amino group, and the β-carbon substituent with the corresponding pharmacophoric groups in D-serine, in order to compare conformational similarity. The more similar the low energy structures are to that of D-serine, the more likely each is to fit the same receptive field as does D-serine. The closeness of pharmacophoric fit is expressed as the sum of the RMS distances, in Å, of each of the four chosen atoms to their counterparts in D-serine. The fitted superimposed structures were then viewed graphically.

Multifit analysis and molecular volume calculations were performed in order to compare the energies that are required and van der Waals volume that result by forcing all analogs to conform to the pharmacophoric conformation of D-serine. All active plus inactive analogs were linked through the carboxyl oxygens, amino nitrogen, and β-carbon substituent by a 20 mdynes/Å spring constant and this "super molecule" was minimized with Maximin2. This procedure was then carried out for all active analogs only. The lowest energy conformation for each analog is calculated that conforms to the receptor geometrical requirements for D-serine activity. The difference between the energy for each analog resulting from the multifit treatment and its corresponding lowest energy conformation found by the conformational search above represents the energy required for each analog to conform to the receptor's geometrical constraints, as dictated by D-serine. Using the MVolume subroutine in SYBYL, volumes were calculated for the entire multifitted structure and the multifitted active compounds. The active volume represents that space that is accommodated by the receptor site. Subtraction of the active volume from the total volume of inactive and active analogs afforded the inactive space, which is detrimental to agonist activity.

Materials. Caesium chloride was obtained from Gallard and Schlesinger (Carle Place, NY); neutral dispase (Type I) from Boehringer Mannheim, and oligo(dT)-cellulose from Collaborative Research. L-Alanine, glycine, L-serine and L-valine were obtained from Pierce (Rockford, IL) and NMDA from Cambridge Research Biochemicals (Wetherston, UK). β-Chloro-D-alanine, β-chloro-L-alanine, *O*-phospho-D-serine, *O*-phospho-L-serine, *O*-methyl-DL-serine, aminomethyl phosphonic acid, glycinamide, glycine hydroxymate, and DL-serine hydroxymate were all obtained from Sigma Chemical Co. (St. Louis, MO); β-fluoro-D-alanine and β-fluoro-L-alanine were the generous gift of Merck, Sharpe and Dohme, (Rahway, NJ). *N*-phosphonomethyl glycine (active ingredient in the herbicide Roundup) was obtained from Monsanto (St. Louis, MO), and glycyl-D-aspartate was the gift of Dr. Peter Petrutz. All other drugs were purchased from Aldrich.

All data are expressed as the mean ± standard error, with the number of estimations in parentheses. The EC_{50} values were determined by the least squares fit of the concentration-response curves to the logistic equation. The 95% confidence intervals were calculated as the logarithm of the EC_{50} values and appear in parentheses.

Results

Activation of the NMDA/Glycine Receptor

At a holding potential of −60 or −70 mV, application of NMDA (100 μM) and glycine (3 μM) evoked an inward current of 53.8 ± 4.7 nA (n = 51 oocytes). No measurable inward current was observed when either glycine, any of the glycine analogs tested, or NMDA were applied alone. In agreement with previous studies (12, 20, 22), the evoked currents were often but not always biphasic. Biphasic currents comprised a rapidly desensitizing initial component followed by a second, slowly developing, "plateau" phase. The presence of the fast component of the evoked current tended to vary between oocytes and was usually more prominent at higher agonist concentrations. When present, this initial rapidly desensitizing component comprised an initial peak, that decayed slowly, with a time constant of approximately 2–4 sec. No attempt was made to further characterize this component of the current in this study. It should be emphasized, however, that this initial peak is not to be confused with the rapidly desensitizing component (time constant of decay, 250 msec) of hippocampal NMDA/glycine currents described by Mayer *et al.* (2). This component is at present unresolvable with our relatively slow perfusion system. As a consequence, the measured amplitudes in all oocytes were determined only once the current had attained a steady state. The shape of the currents, mono- or biphasic, was usually the same for glycine and its analogs in a given oocyte. Fig. 1 shows the ability of two glycine analogs (ACC and (*R*)-(±)-cycloserine) to dose-dependently activate the glycine site in the presence of a fixed NMDA concentration (100 μM). Glycine increased the maximal evoked NMDA response without affecting the EC_{50} of the NMDA concentration-response curve (Fig. 2). This suggests that glycine (and glycine analogs) is permissive for activation of the NMDA receptor rather than acting as an allosteric modulator of NMDA binding. This observation provides the rationale for comparing glycine analogs in the presence of a single fixed concentration of NMDA (100 μM).

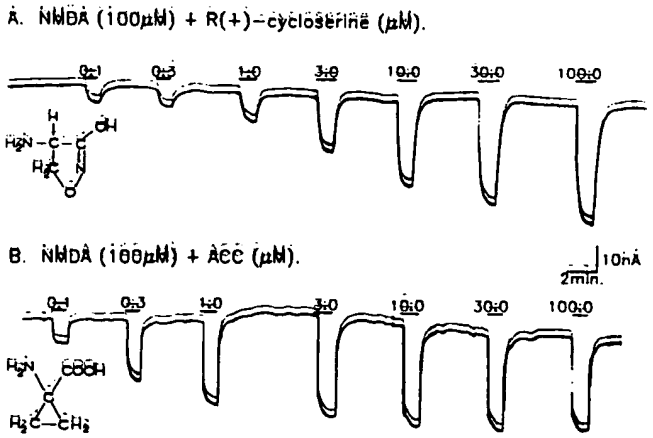


Fig. 1. Concentration-dependent activation of NMDA receptors by the active glycine analogs (*R*)-(±)-cycloserine (A) and ACC (B), determined in two different oocytes that were voltage clamped at −70 mV. Analogs were applied in sequentially increasing concentrations, for the times indicated by the solid bars, in the presence of NMDA (100 μM). Analog-activated currents often comprised two components, particularly at higher concentrations of analog (e.g., 10–100 μM ACC); as a consequence of this observation, all measurements were determined once the currents had attained steady state. The calculated EC_{50} values for (*R*)-(±)-cycloserine and ACC in these experiments were 5.49 and 0.38 μM, respectively. Following the wash-out of each analog, a recovery period of at least 3 min was allowed to elapse before further addition of drug-containing solution. The structure of each analog is shown in the *inset* of each *panel*.

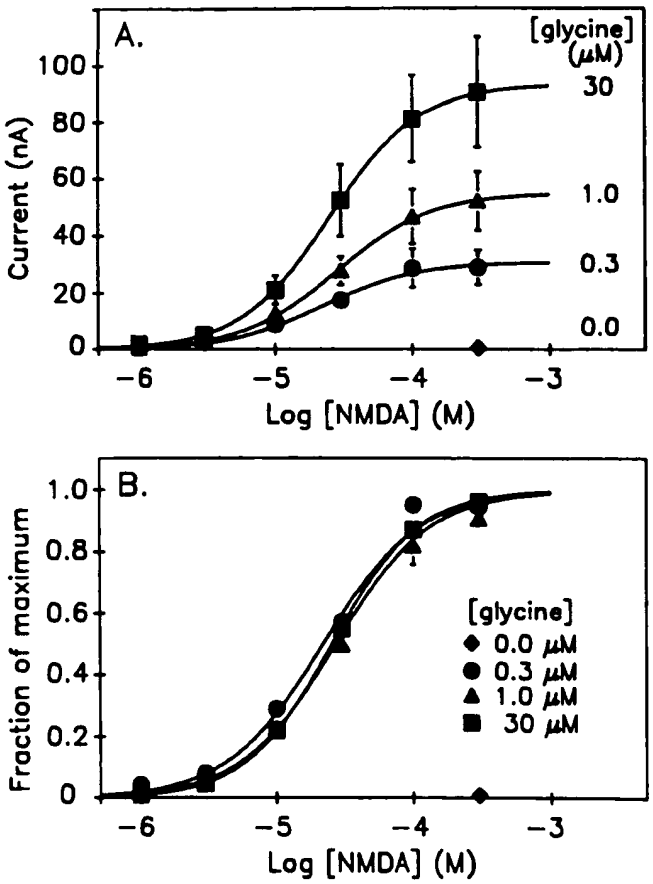


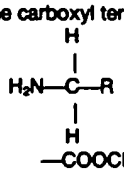
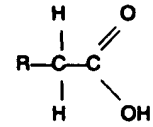
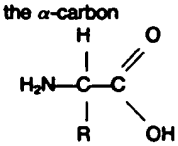
Fig. 2. Glycine increases the maximal induced current but does not shift the EC_{50} of the NMDA concentration-response curve. A, Concentration-response curves were generated in the presence of 0, 0.3, 1.0, and 30.0 μM glycine. Each data point represents the average from 10 oocytes for 0 μM six oocytes for 30.0, and 1.0 μM and four oocytes for 0.3 μM glycine. The *vertical bars* represent the standard error, those data points lacking bars have errors less than the size of the data point. The maximum current amplitudes were 0.6, 29, 53, and 87 nA for 0, 0.3, 1.0, and 30.0 μM glycine, respectively. B, The NMDA responses were expressed as the fraction of the maximal NMDA current for each concentration of glycine. The data indicate that glycine does not alter the EC_{50} of NMDA. The calculated EC_{50} values of NMDA were 23.1 μM (95% confidence limits were 20.8–25.7 μM; $n = 4$), 29.4 μM (24.4–35.6 μM; $n = 6$), and 26.0 μM (24.0–28.2 μM; $n = 6$) for 0.3, 1.0, and 30.0 μM glycine, respectively.

Factors Influencing Activity at the NMDA Receptor

Substitutions at the carboxyl or amino termini. With the exception of (*R*)-(+)-cycloserine (see below), any substitution at either the amino or carboxyl terminus greatly attenuated the ability of the glycine analog to activate the NMDA receptor (Table 1). Removal or partial replacement of the carboxyl terminus (ethanolamine, amino methylphosphonic acid, and taurine) greatly compromised the effectiveness of the glycine analog. Esterification of the carboxyl terminus (glycine methyl and ethyl esters) also markedly attenuated analog activity. Likewise, substitutions on the amino terminus (*N*-methyl-, *N*-acetyl-, and *N*-phosphonomethylglycine and hippuric acid derivatives) or elimination of the amino terminus (acetic acid) were detrimental. Cyclization of the amino terminus (*S*-2-amino azetidine carboxlic acid, pipecolic acid, picolinic acid, and nicotinic acid) also greatly reduced the effectiveness of the glycine analog. This would suggest that, for any activity at the

TABLE 1
Glycine analogs

Each agonist was applied at a single concentration (3 μM) for a period of at least 5 min before the addition of a solution containing 3 μM agonist plus 100 μM NMDA. The data are expressed as mean ± standard error of the percentage of current elicited by 3 μM glycine plus 100 μM NMDA. The number of determinations is three, except where no standard error is shown (two determinations).

	R	Activity
		%
Inactive substitution on the carboxyl terminus		
Glycine methyl ester	—$COOCH_3$	12
Glycine hydroxymate	—CONHOH	7.0 ± 1.1
Glycinamide	—$CONH_2$	5.8 ± 0.7
Serinamide	—$CONH_2$(CH_2OH on αC)	5.8 ± 0.4
DL-Serinehydroxymate	—CONHOH (CH_2OH on αC)	4.3 ± 0.7
Taurine	—CH_2SO_3H	4.1 ± 1.6
Glycine ethyl ester	—$COOC_2H_5$	2.4 ± 3.0
Ethanolamine	—CH_2OH	1.0 ± 1.3
Inactive substitutions on the amino terminus		
N-Methylglycine	—$NHCH_3$	6.4 ± 1.3
N-Acetylglycine	—$NHCOCH_3$	3.7 ± 0.9
N-*p*-Hydroxyhippuric acid	—NHC_6H_5OH	2.5 ± 2.1
2-Hydroxyhippuric acid	—$NHCOC_6H_5OH$	0.8 ± 0.5
α-Hydroxyhippuric acid	—$NHCOC_6H_5$ (OH on αC)	0 ± 0
N-Phosphonomethyl glycine	—$NHPO_3CH_2$	0 ± 0
Acetic acid	—H	0 ± 0
Inactive substitutions on the α-carbon		
O-Phospho-D-serine	—CH_2OPO_3	11.0 ± 3.2
O-Phospho-L-serine	—CH_2OPO_3	2.1 ± 2.5
L-Phenylglycine	—C_6H_5	7
D-Phenylglycine	—C_6H_5	2
L-Valine	—$CH(CH_3)_2$	3.2 ± 1.5
D-Valine	—$CH(CH_3)_2$	1.7 ± 1.3
L-Phenylalanine	—$CH_2C_6H_5$	2.6
D-Phenylalanine	—$CH_2C_6H_5$	7.0
D-3-Hydroxyphenylglycine	—C_6H_4OH	5.0 ± 1.0
2-Aminoisobutyric acid	—$(CH_3)_2$	1.7 ± 1.3
D-Isoleucine	—$HCCH_3C_2H_5$	0 ± 0
L-Isoleucine	—$HCCH_3C_2H_5$	0 ± 0
D-4-Hydroxyphenylglycine	—C_6H_4OH	0 ± 0
O-Methyl-D-serine	—CH_2OCH_3	0 ± 0
Miscellaneous substitutions		
Glycylglutamate		9.1 ± 5.0
D-Pipecolic acid		1.8
L-Pipecolic acid		1.2
$(Glycyl)_3$glycine		1.0 ± 1.3
S-2-Azetidine carboxylic acid		1.0 ± 1.3
Nicotinic acid		0.7 ± 0.9
Glycyl-D-aspartate		0 ± 0
Cystathionine		0 ± 0
Picolinic acid		0 ± 0

glycine site, both carboxyl and amino termini must be free to interact with their appropriate ionic binding sites.

Cyclization of D-serine to D-4-amino-3-isoxazolidone[(*R*)-(+)-cycloserine] sterically fixed the substituted oxygen of serine while removing the carbonyl group of the carboxyl terminus (Fig. 1A). Rather surprisingly, (*R*)-(+)-cycloserine at 3 μM had 27.0 ± 2.4% of the activity of glycine; (*S*)-(−)-cycloserine was virtually inactive (1.7 ± 1.3%). It was considered possible that the response to cycloserine was due in part to its partial decomposition to yield D-serine. This was not supported, however, by the comparison of the p*K* values for both cycloserine and serine, estimated by titration of both compounds with hydrochloric acid. Titration of (*R*)-(+)-cycloserine with HCl showed two pK_a values, one at ~7.5 (presumably the primary amino terminal) and the second at ~4.5, which could reflect ionization of the hydroxyl group. In comparison, the measured p*K* values of serine were consistent with the reported values of 9.1 and 2.2. One initial cleavage product of cycloserine comprises a serine analog with an amide substitution on the carboxyl terminus (serinamide). This substitution was virtually inactive (Table 1). Likewise, glycinamide, glycine hydroxymate, and serinehydroxymate were all inactive analogs. These considerations indicate that the activity of (*R*)-(+)-cycloserine is not due to a breakdown product. The pK_a values of (*R*)-(+)-cycloserine indicate that this compound will exist substantially as a zwitterion at physiological pH and, thus, could interact ionically with the receptor.

Separation of the carboxyl and amino termini. The distance separating the carboxyl and amino residues of the glycine molecule would appear to be critical for activity at the modulatory site. An increase in terminal separation by a single carbon (β-alanine) reduced the analog activity by 96% (Fig. 3). Likewise, a single carbon increase in the interterminal distance of D-serine (DL-isoserine) and D-alanine (DL-3-aminoisobutyric acid or DL-3-aminobutyric acid) resulted in a complete loss of activity. Short chain di- and tripeptides (glycylglycine, glycylglutamate, glycyl-D-aspartate, and glycylglycylglycylglycine) were also without activity at this site (Table 1).

Small-bulk hydrophobic substitutions on the α-carbon. Substitution at the glycine α-carbon with a cyclopropane moiety (ACC) yielded a compound with 73.2 ± 6.8% of the activity of glycine (at 3 μM). ACC proved, however, to have greater potency but slightly lower efficacy than glycine when dose-response data were obtained (see below). D-Methylation of the α-carbon (D-alanine) resulted in a ~38% reduction in activity; the L-isomer was 88% less effective than glycine. 2-Amino-isobutyric acid, an analog of alanine with a methyl substitution at both the L- and D-positions, however, was without activity. Larger hydrophobic, aliphatic, or aromatic substitutions, e.g., an ethyl (D-2-aminobutyric acid), isopropyl (D- or L-valine), butanyl (D- or L-isoleucine), phenyl (D- or L-phenylglycine), methylphenyl (D- or L-phenylalanine), or hydroxyphenyl (D-3-hydroxyphenyl-, D-4-hydroxyphenylglycine) also resulted in analogs with less than 10% effectiveness (Table 1). These results would suggest that steric hindrance around the α-carbon is an important determinent of agonist activity at the glycine site.

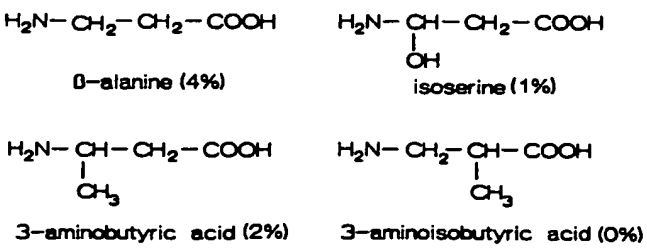


Fig. 3. An increase in the carboxyl-amino interterminal length of active analogs by a single carbon markedly diminishes activity at the glycine modulatory site. Addition of a single carbon into glycine (β-alanine) or D-alanine (3-aminobutyric acid or 3-aminoisobutyric acid) or incorporation of the β-carbon of D-serine into the interterminal chain (isoserine) results in <5% analog activity. The activity of each analog compared with that of glycine was tested at 3 μM and is shown in parentheses.

Hydrogen bonding through α-carbon substituents. Serine. As reported previously, hydroxymethyl substitution of the α-carbon (serine) permits activation of the NMDA/glycine current (1, 12). The D-stereoisomer at 3 μM was 90.0 ± 5.9% (n = 11) as effective as glycine, whereas the L-isomer had only 7.6 ± 2.7% (n = 11) activity. We considered whether the activity of serine could be due to the additional binding of the ω-terminal hydroxyl group to a receptor site usually not involved in glycine binding. This was supported by the observation that an isosteric substitution of the hydroxyl by a methyl group (D-2-aminobutyric acid) eliminated activity (Fig. 4). The binding of the ω-terminal is, therefore, likely to occur through hydrogen bonding, specifically either by proton donation or acceptance.

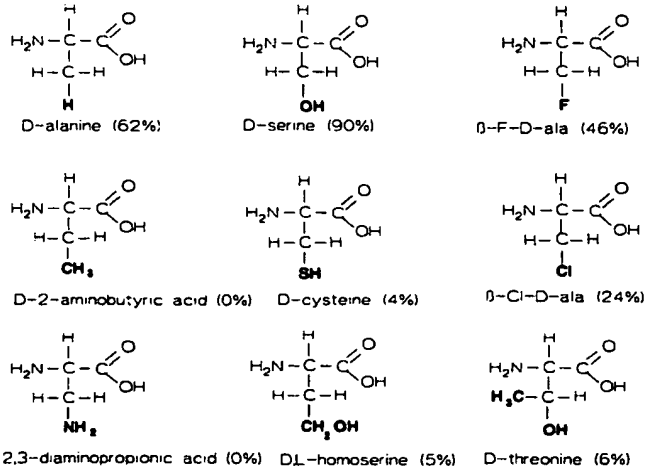


Fig. 4. ω-Terminal substitution of the α-carbon substituent of D-alanine has varying effects on the ability to activate NMDA-induced inward currents. Each analog was tested at 3 μM on at least three oocytes (except D-threonine; n = 2) and compared with the activity of 3 μM glycine. The steric hindrance imposed by α-carbon substitution of glycine (D-alanine) caused a 38% reduction in analog potency. Hydroxyl substitution of alanine (D-serine) partially compensates for this reduced effectiveness, possibly through participation of hydrogen bonding at the ω-terminus. Halogenated compounds (β-fluoro-D-alanine and β-chloro-D-alanine) capable of hydrogen bond acceptance are active, whereas an analog capable only of proton donation (2,3-diaminopropionic acid) was virtually inactive. Likewise isosteric substitution of the hydroxyl group by a methyl, which is incapable of hydrogen bonding (D-2-aminobutyric acid), is inactive. The position and bulk of the ω-terminal substituent are also important in determining analog activity at the glycine modulatory site. Homoserine and threonine, hydroxyl-bearing analogs, and D-cysteine, a thiol-containing analog, all capable of hydrogen bonding, are only ~5% as effective as D-serine. The mean (± SE) currents permitted by each analog were (as a percentage of current at 3 μM glycine) D-alanine, 62 ± 3.8; L-alanine, 12 ± 4.3; D-serine, 90 ± 5.9; L-serine, 7.6 ± 2.7; β-fluoro-D-alanine, 46 ± 4.9; β-fluoro-L-alanine, 0 ± 0; β-chloro-D-alanine, 24 ± 2.1; β-chloro-L-alanine, 0 ± 0; D-cysteine, 4 ± 2.2; D-2-aminobutyric acid, 0 ± 0; 2,3-diaminopropionic acid, 0 ± 0; DL-homoserine, 5 ± 3.0; and D-threonine, 6.

In an attempt to clarify this issue, we investigated a series of analogs having substitutions capable of performing only one of the above interactions.

Proton donors and acceptors. An aminomethyl (2,3-diaminopropionic acid) substitution at the substituted ω-terminus, which if protonated is capable of only proton donation, showed <5% activity relative to glycine (Fig. 4). Compounds containing proton acceptor moieties (β-chloro-D-alanine, β-fluoro-D-alanine) at the β-carbon, however, continued to demonstrate activity, albeit somewhat reduced compared with glycine. The L-stereoisomers were completely inactive at 3 μM. Analog activity was greatly diminished, however, by *O*-methylation and *O*-phosphonation of serine (*O*-methyl-DL-serine and *O*-phospho-D- or L-serine respectively), compounds also capable of only proton acceptance (Table 1). Longer chain analogs containing hydroxyl groups on the substituted residue of the α-carbon (D-threonine and DL-homoserine) and, thus, theoretically able to proton donate or accept, were incapable of permitting NMDA-activated currents (Fig. 4). Likewise, *para*- or *ortho*-hydroxyphenolic substitution of the α-carbon (4-hydroxyphenylglycine and 3-hydroxyphenylglycine, respectively) or thiol substitution (D-cysteine) abolished analog activity. It can be tentatively concluded that the activity conferred on the serine analog is primarily through a proton-accepting action of the analog at a distinct binding site that is located distal to those normally occupied by glycine. Thus, only analogs containing proton-accepting substitutions with minimal steric hindrance, preferably in the D-configuration (D-serine, β-fluoro-D-alanine, and β-chloro-D-alanine), are capable of activating the glycine site.

Dose-Response Relationships

Full concentration-response curves were constructed for those analogs that demonstrated >25% potency compared with glycine, D-serine, D-alanine, ACC, (*R*)-(+)-cycloserine, and β-fluoro-D-alanine (Fig. 5). Dose-response curves using the L-stereoisomers of both alanine and serine were also constructed. The fit of the data to the logistic equation (see Materials and Methods) in all cases was good (Fig. 5). ACC was the most potent of all the analogs tested (EC_{50} = 0.3 μM compared with 0.58 μM for glycine), followed by D-serine, D-alanine, β-fluoro-D-alanine, and (*R*)-(+)-cycloserine (Table 2). The L-stereoisomers of alanine and serine were approximately 20 and 30 times less potent than the corresponding D-isomers. With the possible exception of (*R*)-(+)-cycloserine and β-fluoro-D-alanine, all active analogs were capable of near maximum activation of the current and had Hill coefficients not significantly different from unity (Table 2).

Analogs Tested as Potential Antagonists

A number of glycine analogs were tested for their ability to antagonize the current activated by NMDA (100 μM) and glycine (3 μM); β-alanine, L-serine, L-alanine, taurine, D-alanine, D-serine, *N*-methylglycine, and *N*-acetylglycine (all at 100 μM) and glycine methyl ester, aminomethyl phosphonic acid, L-pipecolic acid, glycylglutamate, glycylglycine, glycylethyl ester, *O*-phospho-D-serine, *O*-phospho-L-serine, picolinic acid, nicotinic acid, glycylglycylglycylglycine, ethanolamine, and glycyl-D-aspartate (all at 3 μM). At the single concentration tested, all analogs were ineffective as antagonists at the glycine site.

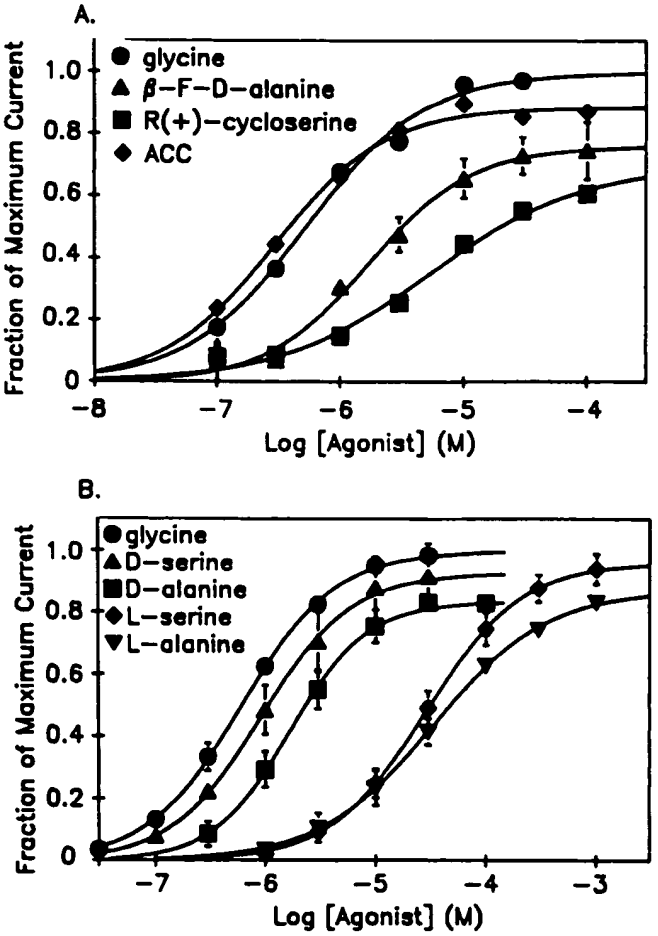


Fig. 5. Concentration-response relationships for glycine and those active analogs having >25% activity at 3 μM. Dose-response curves were constructed as illustrated in Fig. 1. Each *point* is the average response expressed as the fraction of maximum current induced by 30 μM glycine and 100 μM NMDA, determined in each oocyte. With the exception of glycine, each curve was determined from three oocytes; three and seven oocytes were used to construct the glycine curves in A and B, respectively. The *curves* were drawn from the fit of the data to the logistic equation. The *vertical bars* represent the standard error; data points having no bars have their errors within the data point symbol. All analogs were nearly full agonists, with the exception of (*R*)-(+)-cycloserine and β-fluoro-D-alanine. The EC_{50}, efficacy, and Hill coefficient for each analog are shown in Table 2.

TABLE 2

Potency of glycine analogs

EC_{50} and Hill coefficients were calculated from full dose-response curves shown in Fig. 5, as described in Materials and Methods.

	EC_{50}	Efficacy[a]	Hill coefficient	*n*
	μM	%		
ACC	0.30 (0.21–0.39)	89.0 ± 1.80	1.02 ± 0.05	3
Glycine	0.58 (0.46–0.73)	100	1.01 ± 0.06	10
D-Serine	0.93 (0.79–1.22)	94.6 ± 0.88	1.03 ± 0.15	3
D-Alanine	1.70 (1.18–2.69)	85.0 ± 0.42	1.23 ± 0.05	3
β-Fluoro-D-alanine	1.68 (1.56–1.81)	76.0 ± 3.01	1.01 ± 0.1	3
(*R*)-(+)-Cycloserine	5.30 (4.53–6.34)	71.4 ± 7.02	0.71 ± 0.05	3
L-Serine	29.1 (21.9–38.6)	98.9 ± 0.74	1.01 ± 0.46	3
L-Alanine	33.7 (21.0–66.0)	101.2 ± 0.41	0.83 ± 0.09	3

[a] Results are expressed as the percentage of maximum current compared with that evoked by glycine.

Molecular Modelling

In order to model and compare representative active and inactive analogs of similar size possessing an acyclic α-substituent, glycine, D-serine, D-alanine, and β-fluoro-D-alanine were used as active analogs while D-cysteine, D-threonine, *O*-methyl-D-serine, 2-aminobutyric acid, and 2,3-diaminopropionic acid were used as inactive analogs. The presence or absence of activity among the different analogs tested and modelled can be expected to depend upon the characteristics of the β-carbon substituent, because, for the modelled compounds, this represents the only difference in structure. The lowest energy conformation of pharmacophoric atoms in D-serine was nearly identical to that found in β-fluoro-D-alanine, D-alanine (the β-carbon substituent is taken to be H), *O*-methyl-D-serine, and D-cysteine (Fig. 6; Table 3). This is supported by the relatively small RMS fit distances between the pharmacophoric groups among these analogs when compared with D-serine (Table 3). RMS fitting compares only the relative alignment of nuclei, without describing the volume or steric contributions of the analogs. The fit for the lowest energy conformation of the pharmacophoric groups in D-threonine, D-2-aminobutyric acid, and 2,3-diaminopropionic acid to D-serine was considerably less favorable, indicating a lack of conformational similarity.

The greater energy required to force these inactive analogs to conform to the geometry of D-serine suggests that these analogs would present a lower percentage of the total possible conformations in the required form as a time average, resulting in less activity. The result of the multifit analysis shown in Table 3 indicates that D-cysteine, D-threonine, and D-2-aminobutyric acid required approximately 1.5 kcal/mol greater energy input than the remaining analogs in order to align the pharmacophoric groups favorably with the receptor. This is not unexpected, because these same analogs differ significantly from D-serine in their lowest energy conformations. *O*-Methyl-D-serine is an interesting case, because it was inactive pharmacologically (Table 1) yet has a similar low energy conformation to D-serine. The *O*-methyl group, however, is much larger than an hydroxyl and could sterically hinder binding. Indeed, when analogs are forced to conform to the pharmacophoric geometry of D-serine followed by molecular volume calculations, it is obvious that the sulfur of D-cysteine, the β-methyl of D-threonine, the *O*-methyl of *O*-methyl-D-serine, and the α- and β-carbons of D-threonine, 2-aminobutyric acid, and 2,3-diaminopropionic acid present the regions of inactive space presumably not accommodated by the receptor sterically (Fig. 6). The bulk volumes of glycine and ACC would not extend into the inactive space (results not shown).

Discussion

Expression of the NMDA receptor complex in *Xenopus* oocytes provides a suitable model whereby quantitative electrophysiological structure-activity relationships can be deter-

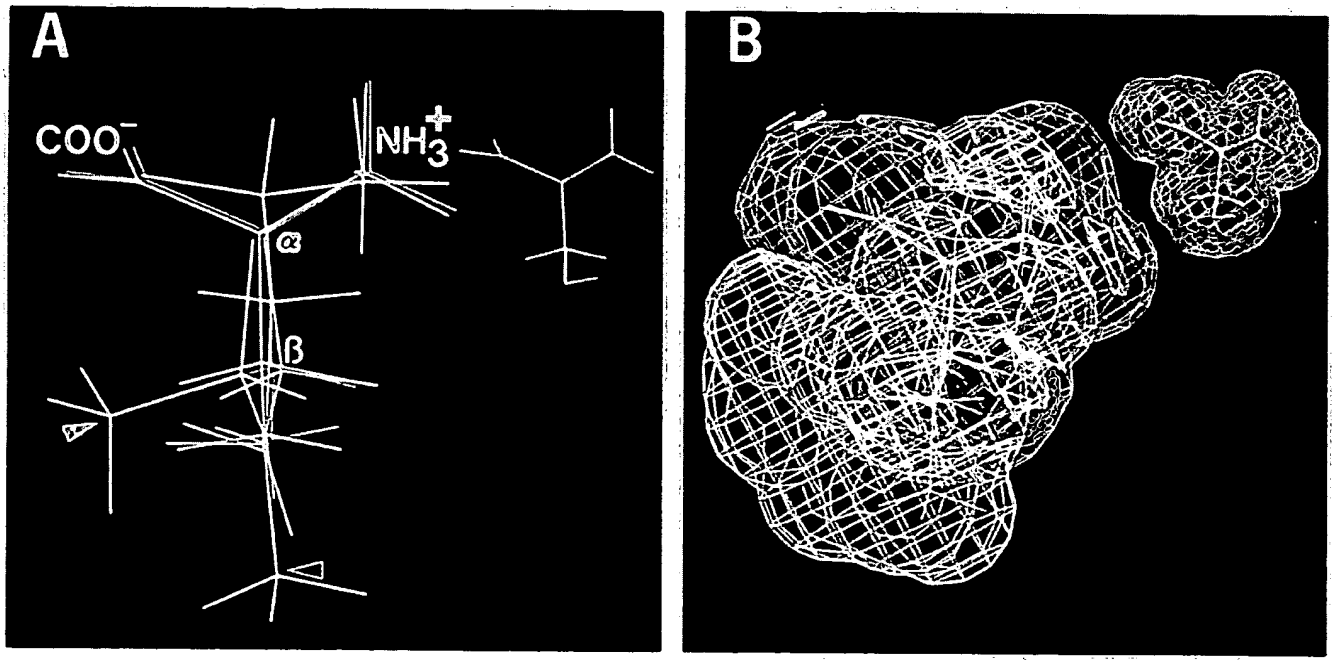


Fig. 6. A, Multifit modelling of active and inactive analogs. All active and inactive analogs listed in table 3 were linked through their pharmacophoric groups and subjected to energy minimization using maximin². The resulting multifitted "supermolecule" is displayed (*main panel*) in which each analog component was forced to conform to the most stable conformation of D-serine. The active analogs are colored red and inactive analogs are yellow. The *upper right small panel* (*red*) shows the superimposition of active analogs D-serine, β-fluoro-D-alanine, and D-alanine; note the near perfect alignment of the three analogs. The superimposition of the inactive and active analogs (*main panel*) demonstrates the close alignment of all carboxyl (COO^-) and amino (NH_3^+) termini and α- and β-carbons. The β-methyl (*solid arrow*) and *O*-methyl (*open arrow*) groups of D-threonine and *O*-methyl-D-serine, respectively, clearly contribute additional steric bulk. B, Construction of pseudoelectronic volume maps for those analogs depicted in A. The *upper right panel* (*red*) demonstrates that volume occupied by D-serine, β-fluoro-D-alanine, and D-alanine. This volume represents the "active" space accommodated by the receptor and can be envisioned as a negative image of the active site of the receptor. "Subtraction" of the active space from the supermolecule represents the inactive space (*blue* or *blue-white*) in the *main panel*, which impairs analog binding to the modulatory site. The steric bulk contribution of the methyl groups in D-threonine and *O*-methyl-D-serine, the α- and β-carbons of D-threonine, 2-aminobutyric acid, and 2,3-diaminopropionic acid, as well as the thiol group in D-cysteine, are seen to project well into this inactive space.

TABLE 3
Conformational requirements of glycine analogs

	Activity	Adaptation to receptor conformation[a]	RMS fit to D-serine[b]
		kcal/mol	Å
D-Serine	Agonist	+2.920	0.0000
β-Fluoro-D-alanine	Agonist	+2.889	0.0411
D-Alanine	Agonist		0.0023
D-Cysteine	Inactive	+4.274	0.1304
D-Threonine	Inactive	+4.551	0.7344
2,3-Diaminopropionic acid	Inactive	+2.842	0.7257
D-2-Aminobutyric acid	Inactive	+4.401	0.6688
O-Methyl-D-serine	Inactive	+2.950	0.0041

[a] The value represents that energy input required to force the minimum energy conformation of each agonist into the steric requirements of the receptor conformation, based on the conformation of D-serine.

[b] RMS distances between pharmacophoric atoms in each analog and those in D-serine. The values are inversely related to the conformational similarity of each analog to that of D-serine.

mined. In agreement with previous observations (12), NMDA, glycine, and all structurally related analogs were unable to evoke measurable currents when applied alone. All observed currents are, therefore, concluded to require the presence of the active glycine analog, i.e., as a coagonist, for the activation by NMDA of the receptor complex. Glycine dose-dependently increases the maximum current evoked by NMDA without measurably affecting the potency of NMDA (Fig. 2). These results support the conclusion from binding studies that the potentiating action of glycine is not mediated by enhancing agonist affinity at the NMDA recognition site (23, 24) and are consistent with the original finding that glycine increases the frequency of opening of NMDA channels (1).

The investigation highlights five structural properties important for the activation of the glycine site. First, all active analogs have a requirement that both carboxyl and amino termini remain unhindered. The ionized hydroxyl group of the carboxyl terminus would appear to be the active constituent, because (*R*)-(+)-cycloserine (having only a free hydroxyl group) was active and those compounds with only free carbonyl groups (serinamide, glycinamide, and serine- and glycinehydroxamate) were inactive. An hydroxyl group per se is insufficient, however, because ethanolamine was inactive. Because both termini will be ionized at neutral pH, it is likely that they bind to positively and negatively charged domains within the receptor. Second, the interterminal distance of these groups would also appear to be critical for receptor activation, because lengthening the chain by even one carbon (e.g., β-alanine) drastically reduced activity. Third, in this study all active analogs had a preference for the D-configuration. Some analogs, however, with minimal activity (i.e., <5%) at the single concentration tested (e.g., phenylglycine, valine, phenylalanine, and pipecolic acid), however, often showed a marginal preference for the L-stereoisomer (Table 1) (cf. Ref. 15). Fourth, those analogs capable of substituting for glycine would appear to belong to one of two classes. The first comprises compounds with a small hydrophobic substitution on the α-carbon (e.g., glycine, alanine, and ACC). These analogs, like glycine, will be zwitterions in neutral pH and are likely to bind only through the ionized carboxyl and amino terminal. ACC has been previously demonstrated to have similar binding characteristics as glycine in inducing [^{3}H]TCP-binding in rat cortical membranes (16). Recently, ACC has been reported to have a partial agonist profile in its ability to promote [^{3}H]MK-801 binding during a 2-hr incubation in rat forebrain membranes (18). In the binding study, ACC was found to have 40% the efficacy of glycine. In our study, we have found ACC to possess a potency almost twice that of glycine but to be nearly a full agonist (~90% efficacy) (Table 2). The measured response in oocytes was determined within 40 sec of the addition of ACC, at the steady state component of the evoked current (Fig. 1). The apparent reduction of efficacy in binding studies may reflect desensitization kinetics at higher concentrations or longer incubation times and warrants further investigation. Whereas the cyclopropane derivative ACC has activity at this agonist site, cycloleucine with its cyclopentane ring has antagonist activity (25). The antagonist activity of cycloleucine may be due to the steric effects imposed by its bulk substituent. Finally, the second category contains those compounds that, like serine, possibly owe their activity to the ability of the β-substituent to hydrogen bond at an additional site of the receptor normally not occupied by glycine. The activity shown by the analog is probably primarily due to hydrogen bond acceptance rather than donation, because compounds possessing β-substituents of either similar bulk to serine but incapable of hydrogen bonding (2-aminobutyric acid) or capable only of hydrogen bond donation (e.g., 1,2-diaminopropionic acid) were inactive.

Results from the molecular modelling suggest that for activity each analog must be able to assume the proper orientation of pharmacophoric functional groups within a reasonable energy window. Assuming that the proper orientation is achievable, the β-carbon substituent must not be too large. The additional capability of the β-carbon substituent to accept an hydrogen bond from the receptor partially compensates for the steric effects of the α-carbon substituent. D-Alanine and ACC do not perturb inactive space and, therefore, the requirement for hydrogen bonding is eliminated. In comparing the active analogs, D-serine meets all of the above requirements and can additionally serve as a hydrogen bond donor, which could stabilize the ligand-receptor complex to an even greater extent. Less potent active analogs such as β-fluoro-D-alanine and β-chloro-D-alanine meet all of the requirements but serve only as hydrogen bond acceptors. Interestingly, halogenated substitutions of large bicyclic compounds promote marked antagonist activity at the glycine modulatory site (11, 13, 26). It is possible that the strong electronegative influence of halogenated analogs will be important in determining analog activity. When considering the inactive analogs, breach of any one requirement abolishes activity. For example, although D-cysteine and D-threonine can adopt conformational similarity to D-serine (Table 3), the steric size of the thiol and methyl group on the β-carbon, respectively, projects well into the inactive space (Fig. 6) and, therefore, those analogs would not be expected to fit the receptor well. The β-carbon substituent of 2-aminobutyric acid can orient with an extra energy input over that of D-serine of 1.48 kcal/mol but is, however, incapable of participating in hydrogen bonding and, therefore, demonstrates little affinity. Likewise, *O*-methyl-D-serine can conform to the required pharmacophoric geometry and can serve as an hydrogen bond acceptor but the *O*-methyl group extends well into the inactive space and is sterically prohibited from binding. D-2,3-Diaminopropionic acid can easily adopt the proper orientation and the β-amino group does not project into inactive space; however, this analog shows no activity. One explanation for this result is

that, because the β-amino group is likely to be protonated at pH 7.4, it would no longer serve as an hydrogen bond acceptor, although it could donate an hydrogen bond.

Comparison with the NMDA and Strychnine-Sensitive Glycine Receptors

It is pertinent to compare those structural features observed in this study at the glycine recognition site with those of both the NMDA recognition site and the strychnine-sensitive inhibitory glycine receptor.

NMDA receptors. A wealth of information exists concerning those structural features important for activation of the NMDA receptor (14, 27). NMDA receptor agonists share many features observed for glycine analogs in this study. Most agonists for the NMDA recognition site comprise a "glycine"-backbone (α-carboxyaminomethyl) plus an additional short chain α-ω interacidic group. The binding of agonists to the NMDA receptor is envisioned as having a three-point attachment of the amino, carboxyl, and ω-acidic termini, although it has been suggested that the ω-terminus of agonists and antagonists may bind to different portions of the NMDA receptor (27). Like agonists at the glycine site, NMDA receptor agonists have a requirement for free ionizable amino and acidic termini. Short chain substitutions at the α-carbon also permit agonist activity at the NMDA receptor. Optimum activity at the NMDA recognition site occurs in analogs bearing either a two-component ("aspartate-length") or three-component ("glutamate-length") chain α-carbon substituent rather than a zero-component ("glycine-length") or one component ("alanine-length") chain. L-Glutamate and glutamate-length agonists have a higher affinity than do aspartate-length agonists (27). Compounds possessing either a carboxyl (COOH) or sulfinate-(SO_2H) group on the ω-terminus of the α-substituent are effective as agonists. An ω-terminal phosphate is generally ineffective for agonist activity at both glycine and NMDA recognition sites but typically confers antagonist activity at the NMDA receptor. Analogs containing ω-terminal sulfonate (SO_3H) groups are effective as NMDA agonists only in glutamate-length α-substitutions. In contrast to NMDA receptor analogs, the presence of an ionizable ω-terminus is not strictly required for glycine analog activity. Stereospecificity of NMDA receptor agonists is not critical for activity. Although both receptors appear to be capable of similar binding interactions, steric influences would appear to be more critical in conferring agonist activity at the glycine site.

Although the actual sizes of the active sites of neither the NMDA site nor the glycine binding site are known, the glycine modulatory site appears to be smaller than the NMDA site. Such a subtle difference in receptor active site volumes may explain the closely related yet differing structural requirements demonstrated by both and it is possible that the third attachment site on the glycine site uncovered in this study is simply not readily accessible to glutamate. Why glycine does not bind to the NMDA recognition site is puzzling and suggests that the attachment of the ω-terminus of NMDA receptor agonist somehow facilitates attachment to the other two binding sites.

Strychnine-sensitive glycine receptor. Relatively little is known concerning structure-activity relationships at the strychnine-sensitive glycine binding site. The actions of a variety of amino acids, however, have been shown to act in a strychnine-sensitive manner at both spinal neurones (28–30) and central neurones (31). These amino acids include L-alanine, β-alanine, taurine, L-serine, D-serine, 2-aminoisobutyric acid, and cystathionine. A number of tentative differences appear from a comparison between this glycine site and the glycine agonist site on NMDA receptors. The carboxyl terminus can be substituted (e.g., taurine), the interterminal length can be longer (β-alanine), the β-carbon substituent can be bulkier and need not participate in hydrogen bonding (2-aminoisobutyric acid and cystathionine), and stereoselectivity is not marked. From this we can deduce that the active site of the strychnine-sensitive glycine site is likely to be larger than that of the glycine site of the NMDA receptor. It is unclear at present whether the three points of attachment observed for both agonists at the NMDA receptor are important for agonist activity at the strychnine-sensitive site.

Glycine as a coagonist. Recent evidence suggests that the family of excitatory and inhibitory amino acid receptors share many structural similarities and are possibly derived from a common ancestry (32). Complementary to both the nicotinic acetylcholine and γ-aminobutyric $acid_A$ receptors, the NMDA receptor complex has a requirement for the binding of at least two agonist molecules for activation. It is likely, however, that the NMDA receptor complex differs from these other receptors in that two structurally related yet *different* agonist molecules are required for receptor activation (i.e., glutamate and glycine). Due to the many common recognition sites shared by agonists of both receptors, we suggest that the glycine binding site was originally derived from the NMDA recognition site or vice versa. Based on reported Hill coefficients obtained from receptor binding assays, it is at present unclear whether the NMDA receptor binds one (33, 34) or more (35) molecules of glutamate. In the latter study a Hill coefficient of 1.22 was found for glutamate potentiation of [^{3}H]MK-801 binding after a 24-hr exposure to L-glutamate. Although these results are intriguing, it is possible that such a lengthy exposure and/or the effects of glutamate at "unprotected" non-NMDA receptors may complicate the interpretation of the obtained Hill slope. At present, we favor the hypothesis of a single glutamate binding site on the NMDA receptor.

We envision, therefore, that the active site of the glycine receptor, like the primary NMDA receptor, is a small pocket containing both positively and negatively ionized groups capable of binding the carboxyl and amino termini of glycine and an additional site capable of proton donation. Thus, only analogs with the correct spatial configuration and binding properties will fit this site. Molecular modelling of these analogs demonstrates a clear profile of structural determinants that appear to be necessary for activity of ligands. The above treatment should prove useful in predicting the relative activity of other potential ligands acting at this site.

The glycine recognition site imparts great functional flexibility to the NMDA receptor. Because neither glycine nor NMDA acting alone can open ion channels, it is reasonable to refer to them as "coagonists" of the NMDA receptor rather than modulator and agonist. It has been difficult to demonstrate the requirement for glycine in intact tissues, presumably due to high levels of contaminating glycine. The competitive glycine site antagonist 7-chlorokynurenic acid (11, 26), however, has been shown to abolish NMDA-induced excitotoxicity in rat cortical cultures (36) and burst firing in rat hippocampal slices (11). In addition, responses to NMDA are blocked by a variety of antagonists with a less specific action at the glycine

site (37–40). These data would suggest that glycine is required in some neuronal preparations as well as in oocytes (12) and dialyzed neurones (3, 13). A major unanswered question is whether this agonist site is saturated with glycine under all conditions, because the EC_{50} for glycine in oocytes, dialyzed neurones, and isolated washed membrane preparations is 200–500 nM (1–3, 7, 12, 13, 16) whereas the glycine concentration in (human) cerebrospinal fluid is estimated to be 7 μM (41). On the other hand, the EC_{50} for glycine potentiation of NMDA-induced excitotoxicity is in the range of 20–30 μM (36), which raises the possibility that the measured affinity of the recognition site for glycine may be influenced by either metabolic or uptake mechanisms (or both) in intact neurones.

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